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HIV-1 and the Nucleolus: A Role for Nucleophosmin/NPM1 in Viral Replication: A Dissertation

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HIV-1 AND THE NUCLEOLUS: A ROLE FOR NUCLEOPHOSMIN/ NPM1 IN VIRAL REPLICATION

A Dissertation Presented

By

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Interdisciplinary Graduate Program

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DEDICATION

To my husband, Mickaël;

To my Mom, Martha;

To my brother, Chris.

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The journey from initial consideration through the actual achievement of a doctoral thesis is not one accomplished alone, it takes a community of people to guide, encourage and support you along the way. I have had the privilege to be a part an amazing community throughout my years of graduate school that has contributed tremendously to my growth, both professionally and personally, and I am truly grateful.

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ABSTRACT

The nucleolus is a plurifunctional organelle with dynamic protein exchange involved in diverse aspects of cell biology. Additionally, the nucleolus has been shown to have a role in the replication of numerous viruses, which includes HIV-1. Several groups have reported HIV-1 vRNA localization within the nucleolus. Moreover, it has been demonstrated the HIV-1 Rev protein localizes to the nucleolus and interacts with nucleolar proteins, including NPM1. Despite evidence for a nucleolar involvement during replication, a functional link has not been demonstrated. I investigated whether intron-containing vRNAs have a Rev-mediated nucleolar localization step prior to export. Furthermore, I examined whether NPM1 mediates Rev nucleolar localization, participates in Rev function, and/or post-transcriptional events during viral replication. I used coupled RNA fluorescence *in situ* hybridization and indirect immunofluorescence to visualize intron-containing vRNA relative to the nucleolus in the absence or presence of Rev expression. An RNAi-based approach was used to examine the role of NPM1 in Rev function and viral replication in cell lines and primary human macrophages. My research findings support a model for a Rev-independent nucleolar localization step of intron-containing vRNA prior to export. Intriguingly, my results also suggest NPM1 does not participate in Rev nucleolar localization or Rev-mediated vRNA export, as previously proposed. Rather, my findings support a novel role for NPM1, the cytoplasmic localization and utilization of a select class of Rev-dependent vRNAs. Collectively, my

findings provide novel insight for a functional role of the nucleolus and NPM1 in HIV-1 replication, which enhances our current understanding of HIV-1 biology.

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LIST OF ABBREVIATIONS

Aa	amino acid
ADC	AIDS dementia complex
AF	AlexaFluor
AIDS	acquired immunodeficiency syndrome
AML	acute myeloid leukemia
ARM	arginine rich motif
AZT	zidovudine
CA	capsid
CAT	chloramphenicol acetyltransferase
CCR5	C-C chemokine receptor type 5
CDC	Center for Disease Control
CLEC2D	C-type lectin domain family 2 member D
Cpm	counts per minute
CRM1	Chromosome Maintenance 1
CTL	cytotoxic T cells
CXCR4	C-X-C chemokine receptor type 4
CypA	Cyclophylin A
DC	dense fibrillar component
dsDNA	double stranded DNA
ELISA	enzyme-linked immunosorbent assay

Env	envelope
ER	endoplasmic reticulum
ESCRT	endosomal sorting complex required for transport
FC	fibrillar center
FISH	fluorescent *in situ* hybridization
Gag	Group specific antigen
GC	granular component
GFP	enhanced green fluorescent protein, green fluorescent protein
Gp	glycoprotein
gRNA	genomic RNA
GTP	Guanosine-5'-triphosphate
HAART	Highly Active Antiviral Therapy
HIV-1	Human Immunodeficiency Virus Type I
hnRNP	heterogeneous nuclear reibonucleoprotein
hPIMT	human peroxisome proliferator-activated receptor-interacting protein with methyltransferase
hRIP	human Rev-interacting protein
IF	indirect immunofluorescence
Ig	Immuglobulin
IN	integrase
kD	kilodalton
LAV	lymphadenopathy-associated virus

LTR	long terminal repeat
MA	matrix
MATR3	Matrin 3
MDM	monocyte-derived primary human macrophages
mRNA	message RNA
NC	nucleocapsid
Nef	negative regulatory factor
NES	nuclear export signal
NLS	nuclear localization signal
NNRTI	non-nucleoside reverse transcriptase inhibitors
NoLS	nucleolar localization signal
NPM1	Nucleophosmin, nucleolar phosphoprotein B23, numatrin, B23, NPM
NRTI	nucleoside reverse transcriptase inhibitor
Nt	nucleotide
Nup	nucleoporin
p6	protein of 6 kilodaltons
Pbs	primer binding site
PCP	*Pneumocystis carnii* pneumonia
PI	protease inhibitors
PIC	preintegration complex
Pol	polymerase
PR	protease

PSF	Polypyrimidine tract bonding protein associated splicing factor
qPCR	quantitative real-time polymerase chain reaction
RBD	RNA binding domain
Rev	regulator of virion expression
RNAi	RNA interference
RRE	Rev response element
rRNA	ribosomal RNA
RT	reverse transcriptase
RTC	reverse transcriptase complex
Sa	splice acceptor site
Sd	splice donor site
SEM	standard error of the mean
siRNA	small interfering RNA
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
SP	spacer peptide
SU	surface envelope glycoprotein gp120
TAP	Tip-associated protein
TAR	trans-activating response element
Tat	trans-activator of transcription
TM	envelope transmembrane glycoprotein gp41
tRNA	transfer RNA

Vpr	viral protein R
Vpu	viral protein U
vRNA	viral RNA
VSV-G	vesicular stomatitis virus envelope glycoprotein G

CHAPTER I: INTRODUCTION

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1.1 HIV-1 and AIDS: A Global Epidemic

1.1.1 Discovery

Human Immunodeficiency Virus Type I (HIV-1) is the etiological agent of acquired immunodeficiency syndrome (AIDS) and responsible for more than 30 million deaths worldwide (318). The beginning of the HIV-1/AIDS epidemic is marked as June 5, 1981 when the Centers for Disease Control (CDC) published a report observing cases of the lung infection *Pneumocystis carinii* pneumonia (PCP) in a small population of young, previously healthy, homosexual males (54). The report noted the incidence of PCP was exceedingly rare and usually manifested in severely immunocompromised individuals. Within weeks, increased accounts of other unrelated opportunistic infections, such as Kaposi Sarcoma, mucosal candidiasis and chronic ulcerating lesions by herpes simplex virus, were being reported in men across metropolitan areas that were also displaying similar unexplained immunological defects, suggesting an outbreak of a common, transmissible cause (53, 129, 164, 219, 290). Soon after, the new acquired immunodeficiency disease was also observed in the larger population, such as in heterosexuals, hemophiliacs (51, 219) and later in women and children (52, 55), demonstrating more than one population was at risk.

The observations that patients receiving blood transfusions and the sexual partners of individuals with AIDS, both who were previously healthy, were then later seen with AIDS symptoms, clearly demonstrated the causative agent was transmitted through blood

and sexual activities and its persistence and spread throughout the larger population suggested a virus. Luc Montagnier and colleagues from the Pasteur Institute were the first to publish on a virus isolated from AIDS patients, a retrovirus which they termed lymphadenopathy-associated virus (LAV), and suggested that it might be the cause of AIDS (15). Shortly after, Robert Gallo and his research group at the National Cancer Institute isolated and described what they designated as human T-lymphotropic virus type III (HTLV-III) as the probable cause of AIDS (120, 261, 280, 284). Meanwhile, Levy and colleagues also isolated a retrovirus which they named AIDS-Related virus (236). After much debate and sequence verification, it was clear that each group identified a variant of the same retrovirus which was assigned the single name, Human Immunodeficiency Virus (67). While Gallo was not the first to isolate HIV-1, he was the first to establish an enzyme-linked immunosorbent assay (ELISA) that enabled researchers and physicians to test individuals for HIV-1 infection (120, 280).

1.1.2 Disease progression

There are three major routes an individual may contract HIV-1; through sexual intercourse; exchange of contaminated blood, such as through intravenous drug use or blood transfusions; and mother to child, either intrapartum, perinatally, or through breast milk. Although there are several modes of transmission, the subsequent course of HIV-1 infection if left untreated remains the same. HIV-1 targets and depletes CD4 positive (CD4+) cells of the immune system and is characterized by three stages of progression, acute, chronic and AIDS followed by death (Figure 1-1) (5, 87, 88, 303).

The acute phase begins with the primary infection and continues through the development of an HIV-1 specific immune response. Upon infection, the virus quickly replicates, disseminates through and establishes a latent reservoir in the lymphoid tissues and causes a sharp decrease in CD4+ T cells (88). Although virus populations are found throughout the blood and lymph tissues, it is the gut-associated lymphoid tissue (GALT) that is the most affected, seeing a massive and permanent depletion of CD4+ T cells during this time (37, 88, 303). An infected individual may experience mononucleosis-like symptoms such as fever and pharyngitis, also referred to as acute HIV-1 syndrome, within 3-6 weeks following infection. This corresponds to the detectable specific antiviral immune response (68, 118). These antiviral immune responses are associated with the observed decline in viral load and a temporary rise in the number of CD4+ T cells primarily due to the activation and expansion of CD8+ cytolytic T cells (CTL) (28, 68, 145, 253).

Following the induction of the immune response there is a relatively long period in which an infected individual appears asymptomatic, termed the chronic phase. There is an initial stabilization of viral load, called the viral set point, which can be correlated to the time interval from infection to development of opportunistic infections (263) as well as a modest, delayed and temporary recovery of CD4+ T cells from their lowest point. This increase slowly declines over time, an average of 10 years, until the progression to AIDS (88). Although sometimes referred to as clinical latency period, this implies the virus is dormant. In actuality there is continual, rapid viral replication in the progression to disease and is characterized by a remarkable increase of genetic variation in the virus

population and high level of immune activation of CD4+ and CD8+ T cells (88, 142). Eventually, the unstable equilibrium between viral replication and T cell activation, death and renewal breaks down. When CD4+ T cells reach approximately 500 cells/μL certain constitutional infections and malignancies manifest, such as oral lesions, carcinomas of the skin and reactivation of latent herpes zoster virus, all which mark the progression to the final disease state, AIDS (103).

Once the CD4+ cells counts fall below 200 cells/μL, the individual is considered in the AIDS phase of the disease. At this stage, an individual is susceptible to opportunistic infections and neoplasms, such as *Pneumocytisis carinii* pneumonia, Kaposi's sarcoma and herpes simplex virus, as described in the first cases of AIDS. Patients may also develop HIV-1 induced encephalopathy known as AIDS dementia complex (ADC) (94, 103). The viral load continues to rise and the CD4+ T cell counts continue to decrease until virtually all CD4+ T cells are lost, leading to eventual death due to opportunistic infection or cancer.

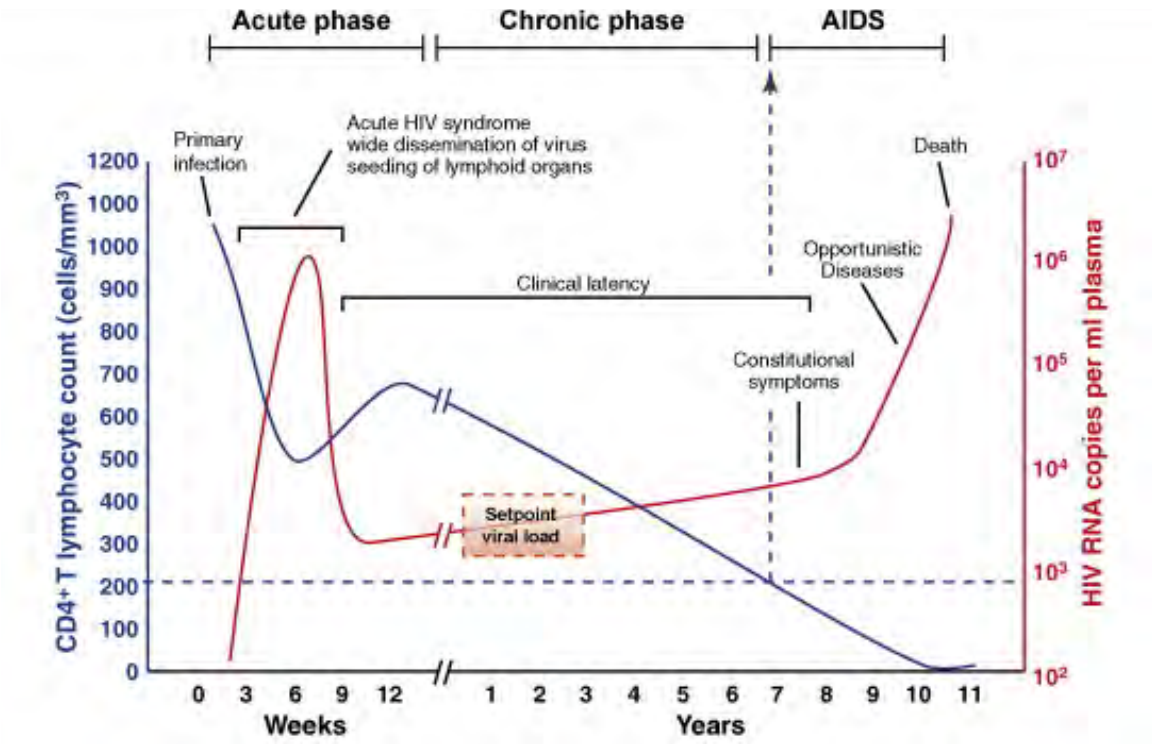

Figure 1-1. Pathogenesis of HIV-1 infection. The typical course of infection in an untreated individual. Primary infection causes rapid viral replication (red), spread of the virus throughout lymphoid tissues where the virus establishes a reservoir and causes a significant decrease in CD4+ T cells (blue). Flu-like symptoms (Acute HIV syndrome) appear with the development of a HIV-1 specific immune response. Next, is the chronic phase where a drop and stabilization of viral load (setpoint viral load) and slight increase in CD4+ T cell counts is seen, lasting several years. Progressively, the number of CD4+ T cells slowly drops and viremia rises. When CD4+ T cells decline to 500 cells/mm^3, constitutional symptoms begin. Progression into AIDS is associated with a CD4+ T cell count of ±200 cells/mm^3 (blue dotted line) and opportunistic disease manifestation, leading to death. *Adapted from An Ping and Cheryl A. Winkler. Trends Genet. (2010).*

1.1.3 Antiviral therapy

The development of antiviral therapies has allowed for long-lasting suppression of viral replication, thus greatly increasing the longevity spent within the clinically latent period of infection and the quality of life of infected individuals. There are now numerous drugs that exist in a variety of categories based on their enzymatic and/or cellular targets. The first class of therapy introduced for the treatment of HIV-1 infection was a nucleoside reverse transcriptase (RT) inhibitor (NRTI), called zidovudine (AZT) in 1987 (96). AZT and other first class RT inhibitors were administered to patients as a monotherapy and were able to prolong life for 6-18 months (81). However, the therapeutic potential of these drugs alone proved ineffective, as they displayed high toxicity and the emergence of viral resistance (94).

The next class of HIV-1 antiviral drugs approved was the PIs (protease inhibitors), the first being Saquinavir in 1995, which revolutionized how antiretroviral therapy was administered. Introduction of PIs began the era of Highly Active Antiviral Therapy (HAART) in which using drugs from different classes in combination proved to drastically reduce viral replication and the incidence of opportunistic infections in AIDS patients (81, 252). A year later, non-nucleoside RT inhibitors (NNRTIs) were introduced further expanding the HAART repertoire. Over the years, treatments have improved not only because of the increased efficacy of the drugs available, but also because the newer compounds have far less toxicity and dosages have been simplified. In some cases a combination of drugs is made into a single pill given once daily, which is more practical,

allowing for easier adherence to the treatment regime, leading to the transformation of AIDS to a chronic disease.

Drug resistance is one of the frequent causes of treatment failure. Viral resistance to drug therapy has been reported for every antiviral drug available (84) and therefore emphasizes the critical need for new HIV-1 drug discoveries, and more importantly, development of a HIV-1 vaccine. The extraordinary viral diversity and rapid error-prone replication of the virus are not only reasons for drug resistance but are considerable challenges faced in the development of a vaccine. Incomplete understanding of HIV-1 immunopathogenesis as well as the fact that HIV-1 targets the immune system and the very cells that prevent or clear infections further complicates vaccine strategies.

Despite these obstacles, there have been four vaccines that have been brought from concept to human clinical efficacy trials to date (222), unfortunately there still is no vaccine available and only one of the trials has actually shown promise with a modest protection against infection. The first trial was completed in 2003 with two parallel vaccine products (AIDSVAX B/E and AIDSVAX B/B) of HIV-1 envelope proteins and failed to show any effectiveness (107, 258). The second trial, known as the STEP trial, used an attenuated adenovirus vector approach, fared worse and was stopped prematurely in 2007 due to evidence that the vaccine actually increased susceptibility of HIV-1 infection in some populations (102). Optimism of reaching an HIV-1 vaccine increased upon announcement of results in the RV144 Thailand community-based trial that showed a 31.2% protective effect against infection using a canarypox vector and recombinant glycoprotein 120 (gp120) approach (222). However, researchers and physicians are still

far from a safe and effective prophylactic vaccine for HIV-1. This is exemplified by the April 25, 2013 announcement from the US National Institute of Allergy and Infectious Diseases that it has halted the most recent vaccine efficacy trial, HTVN 505, due to its lack of ability to prevent infection or reduce viremia (239).

Irrespective of this disappointment, the universal necessity for a vaccine continues to drive the coordinated international effort towards its goal of HIV-1 eradication. Scientists are confident that a cure for HIV-1 can be reached. To date there are three cases in which a 'functional' cure of HIV-1 has been documented, meaning a previously HIV-1 positive individual has no to barely detectable levels of the virus within their blood or other latent reservoirs after an extended period of time and no longer requires antiretroviral therapy. Normally, once antiviral therapy is ceased virus replication rebounds and if not controlled, disease progression continues until death.

The first case of a functional cure was Timothy Brown, known as the Berlin patient, in 2009. Brown was living with HIV-1 for 10 years and on antiretroviral therapy before he was diagnosed with acute myeloid leukemia (AML), a cancer of the myeloid cell lineage causing rapid growth and accumulation of abnormal white blood cells in bone marrow. To treat the cancer, Brown underwent a hematopoietic stem cell transplant using a matched donor that also had the rare, homozygous genetic mutation CCR5Δ32 in the coreceptor utilized by macrophage-tropic HIV-1 to enter a cell. This mutation renders an individual highly resistant to infection and is present in only 1-2% of Caucasians (163, 172). Since his transplant, researchers and physicians have been unable to detect levels of HIV-1 in blood or other tissues and he remains off antiviral

therapy (163, 172). While this procedure is not possible for widespread use, this provides proof-of-concept that a cure is attainable.

Further promise towards HIV-1 eradication came from two separate reports within weeks of each other in March of this year. Researchers at the Conference on Retroviruses and Opportunistic Infections (CROI) announced a case of a functional cure in a 26 month old infant that began antiretroviral therapy within 30 hours of birth and continued until 18 months of age. Although at age 26 months HIV-1 DNA was detectable (4 copies/million), there were no detectable levels of plasma viral load or HIV-1 specific antibodies by standard clinical methods, and therefore the infant was recognized as functionally cured (83). This case strongly argues for aggressive and early treatment in infants to inhibit latent reservoir establishment as a cure, and is currently being pursued in a clinical trial. This is a difficult but potentially achievable solution that can be applied with current procedures used globally to reduce mother-to-child transmission.

Aggressive, prolonged antiviral therapy early in infection has also been shown to provide a functional cure in adults for the first time. French researchers have reported 14 individuals that were treated within weeks of initial infection for an average of 3 years now have normal CD4+ T cell counts, low viral load, and have been off antiretroviral therapy for an average of 7 years (276). Although extremely encouraging, evidence suggests that only 10-15% of adults may be cured by this approach (172, 276), and only if exposed individuals actually receive immediate treatment. This presents a method that

is neither relevant for those with chronic infection nor realistic to those affected the greatest, individuals from developing countries.

1.1.4 Global Impact

The global need for an HIV-1 vaccine and a cure is obvious. There are approximately 34 million people currently living with HIV-1 worldwide and it has caused more than 30 million deaths since its discovery (318). Moreover, chronic management of HIV-1 has not been realized in mid to low-income countries. More than 97% of the 2.5 million new infections occur in these nations, with only 7% in developing countries having access to antiretroviral drugs and only 20% worldwide being reached by prevention programs (319, 325). Restrictions such as poor governmental support, limited health infrastructure, licensing and cost of available drugs, have all contributed to the inability to reach those needing treatment in these regions (291). Joint United Nations Programme on HIV/AIDS (UNAIDS) has a new “15 by 15” campaign, with a goal to provide 15 million people in developing countries with treatment by 2015 (319). Other extraordinary, organized initiatives targeting underserved populations provide encouraging advances towards the goal of global access for treatment and preventative care of HIV-1. Progression towards this objective is reflected in the 20% decrease in reported newly infected people since its peak in 2001, with the sharpest declines being in the Caribbean (42%) and sub-Saharan Africa (25%) (319), but is far from being fully realized. This provides further evidence for the need to push for even greater scale-up initiatives, development of alternative treatments and preventative measures, such as

microbicides, circumcision and pre/post exposure prophylaxes. Furthermore, equally if not more importantly, increased support in basic science research to be able to fully unravel the complexity of HIV-1 pathogenesis and supply the world with a much needed cure.

1.2 The HIV-1 Replication Cycle

HIV-1 is part of the *Retroviridae* family of viruses, a family with a unique life cycle that differentiates them drastically from other viruses. Replication is an intricate, multiple step process that is regulated by both viral and cellular proteins which confers the ability to perform a reverse flow of genetic information from RNA to DNA and insertion of the proviral genetic information into the host genome. Integration into the host genome allows the virus to maintain a persistent infection irrespective to the host immune response. The HIV-1 life cycle can be divided into a series of events occurring at two stages, early or late, based on the relationship of the steps to viral DNA integration into the host genome (Figure 1-2). Replication begins with the attachment of the viral surface envelope glycoprotein gp120 (SU) to the host cell surface receptor CD4, an Immuglobulin (Ig)-like protein found on a subset of T-cells, macrophages and dendritic cells. Binding of gp120 to CD4 induces a conformational change and enables its subsequent binding to one of two co-receptors; host seven-transmembrane chemokine receptors CXCR4 or CCR5. Further structural changes occur upon co-receptor binding in both gp120 and the envelope transmembrane glycoprotein gp41 (TM). This promotes viral envelope and cell plasma membrane fusion and allows the release of the viral core

into the host cell cytoplasm (63, 281). Once in the cytoplasm the viral core, comprised of viral and host proteins surrounded by a capsid protein (CA) hexameric lattice, undergoes uncoating, a process that is poorly understood. During this step CA is shed, in part or in total, to reveal the reverse transcriptase complex (RTC) made up of the genomic RNA (gRNA), the viral proteins matrix (MA), nucleocapsid (NC), reverse transcriptase (RT), integrase (IN), and viral protein R (Vpr). Next, reverse transcription of the gRNA into linear double stranded DNA occurs (101). As uncoating and reverse transcription of the RTC are taking place, the complex is being transported towards the nucleus, first by associating with actin then by interactions with the microtubule network (40, 44, 221). Completion of reverse transcription results in the formation of the large nucleoprotein complex known as the preintegration complex (PIC) which is then imported from the cytoplasm through the intact nuclear envelope to the nucleus using a combination of viral and cellular proteins. The precise mechanism, along with viral and cellular proteins involved in this step remains controversial (39). Once in the nucleus, IN mediates integration of the viral DNA into the host genome, where it is known as the provirus, allowing for the permanent propagation of virus within the viable cell (111).

Following the integration of the viral genome is the late stage of viral replication. The late stage begins with cellular RNA Polymerase II driving transcription of the viral DNA from the promotor located in the 5' long terminal repeat (LTR). Transcription also requires the viral protein Tat for enhanced rate, processing and elongation of the viral transcripts (111). The vRNA that is produced is then either completely spliced by the cellular splicing machinery and exported to the cytoplasm through the typical TAP (Tip-

associated protein) mRNA export pathway, or is partially spliced or remains full-length (unspliced) and exported to the cytoplasm by the viral protein Rev through the CRM1 (Chromosome Maintenance 1) pathway typical for cellular small nuclear RNAs (snRNAs) and ribosomal RNA (rRNA) (72). After nuclear export viral RNAs (vRNAs) are translated, followed by viral protein and gRNA transportation to sites of virus assembly at the plasma membrane. The functional domains of the viral polyprotein Gag coordinate particle assembly and release. Briefly, matrix (MA) targets and binds the polyprotein to the membrane as well as promoting assembly and Env incorporation, capsid (CA) mediates Gag:Gag interactions and Gag-Pol incorporation with nucleocapsid (NC), which also facilitates dimerization and packaging of gRNA, while p6 promotes Vpr (viral protein R) incorporation into the virus and recruitment of the cellular machinery needed for virus budding and release (112). As the virion buds and is released from the cell membrane it undergoes its final step, maturation. Maturation is a morphological rearrangement that involves the proteolytic processing of the Gag and Gag-Pol polyproteins by the viral protein protease (PR) and once completed the virus is infectious and ready to begin another round of replication in a new target cell (111, 329).

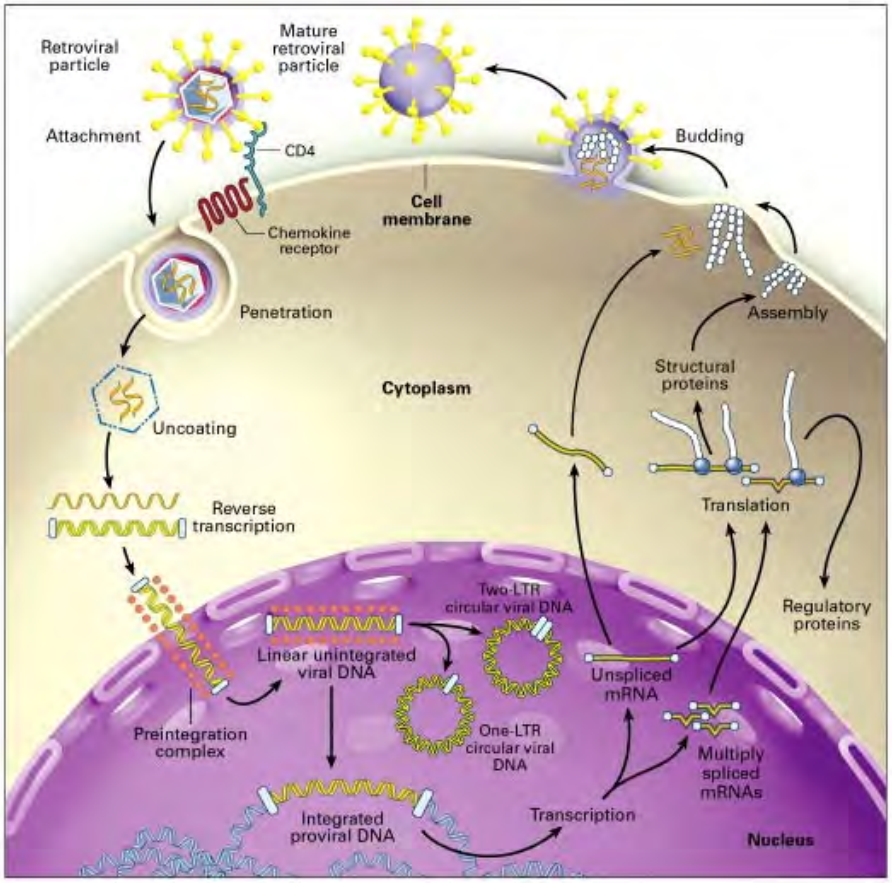

Figure 1-2. The HIV-1 replication cycle. HIV-1 replication is divided into two stages classified by the relationship of the steps to viral DNA integration: early (preintegration) and late (postintegration). Early stage begins with viral attachment followed by viral core penetration and uncoating, reverse transcription of the vRNA into DNA, nuclear import of the preintegration complex and concludes with incorporation of the viral DNA into the host genome. The late stage consists of viral gene transcription, export of unsliced, partially spliced and fully-spliced vRNA to the cytoplasm, translation, viral particle assembly, budding and release from the host cell and finally, maturation of the virion. *Reproduced with permission from Furtado, MR, et al. N. Engl. J. Med. (1999), Copyright Massachusetts Medical Society.*

1.3 The HIV-1 Genome: Organization and Viral Proteins

1.3.1 Virion and Genome Organization

An infectious, mature HIV-1 virion is a sphere of approximately 125 nm in diameter containing a protein dense conical viral core (Figure 1-3) (38, 345). The spherical outer shell is an envelope lipid membrane bilayer containing 8-10 trimers of non-covalently interacting Env glycoproteins SU (gp120) and TM (gp41) and is supported by an inner layer of MA (38, 345). Moving inward, the interior of the virion is comprised of protease (PR), cellular cyclophylin A (CypA) and the viral core (123, 322). The viral core is a protein complex of predicted fullerene conical shape (124) with approximately 250 hexameric and 12-15 pentameric rings of CA making up the outer lattice shell (123) that encases the inner core viral proteins RT, IN, p6, PR, Nef (negative regulatory factor), Vif (viral infectivity factor) and Vpr (38, 123, 183, 322). Also making up the inner core is NC, which is responsible for the packaging of the viral genome, a condensed dimer of two copies of the HIV-1 genome (123, 234, 322).

The HIV-1 genome is a positive-sense, single-stranded RNA and is packaged as a dimer (116). The genome is approximately 9.7 kb in length, measuring from the 5’ LTR (long terminal repeat) to the 3’ LTR (116) and contains all the necessary information for infectious virus production (Figure 1-3). The 5’ UTR (untranslated region) does not encode for any viral proteins but does contain several sequence elements needed for viral replication, including: 1) the trans-activating response element (TAR), a RNA hairpin that Tat binds to for transcription initiation, 2) the primer binding site (pbs) needed to initiate reverse transcription, 3) the 5’ Poly A Stem Loop, a region used to stabilize the

$tRNA^{Lys}$ primer, 4) the dimerization site, which includes the self-complementary DIS (dimerization initiation sequences) that form a “kissing loop” to facilitate RNA dimer incorporation into the virion, 5) the major splice donor site (MSD), which is the sequence used to produce all spliced RNAs and finally 6) the translation initiation codon for Gag (111, 116). Found downstream are the nine overlapping open reading frames (ORFs) which encode for the 15 viral proteins. HIV-1 is able to produce all proteins from a single transcript through the utilization of alternative splicing by the selection of one of 5 splice donor and more than 10 splice acceptor sites (sd and sa, respectively) (266), and the production of polyprotein precursor translation products. Unspliced RNA produces the Gag and Gag-Pol polyproteins that during virion maturation become cleaved to generate MA, CA, NC, p6, PR, RT and IN proteins. RNAs that are singly spliced produce the proteins Vif, Vpr, Vpu (viral protein U), and the Env polyprotein which is later cleaved by cellular proteases to produce SU and TM proteins. Completely spliced RNAs give rise to Nef, Tat, and Rev viral proteins. Rev is required for the expression of the unspliced and singly spliced RNAs by mediating their export to the cytoplasm through sequence-specific binding to a stem-loop structure, termed the Rev response element (RRE), located within the *env* coding region. Another element found within the genome is the polyadenylation signal used to generate the 3’ end of the transcripts which is located in the 3’ LTR. The complexities of the genome are also reflected in the functional roles of the individual proteins themselves and are categorized based on such, into structural, enzymatic, regulatory or accessory proteins.

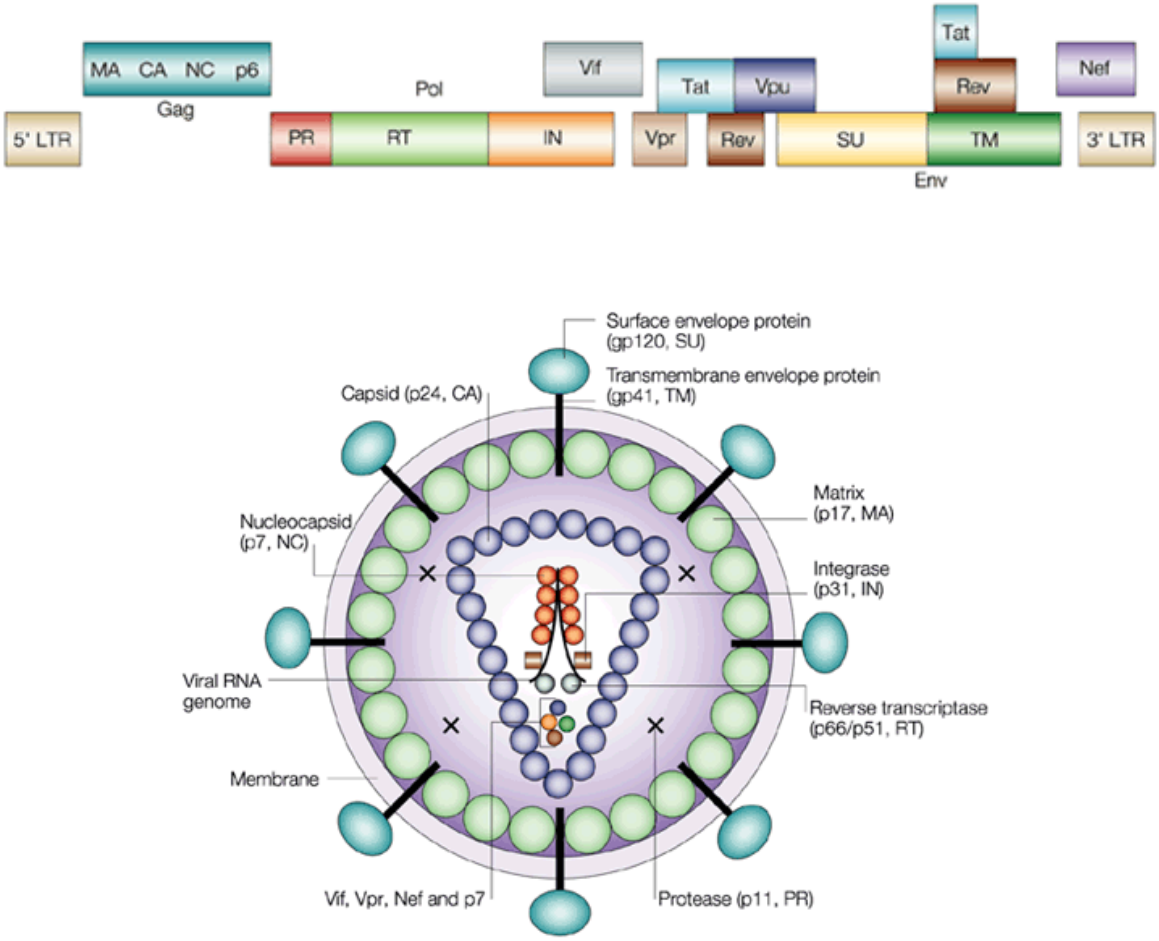

Figure 1-3. The HIV-1 genome and mature virion organization. *Adapted from H.L. Robinson Nat. Rev. Immunol. (2002).*

1.3.2 Structural proteins

The structural proteins are comprised of the Gag and Env polyproteins and their cleavage products. Gag is synthesized on cytosolic polysomes as a 55 kD polyprotein precursor ($Pr55^{Gag}$) comprised of four domains: matrix (MA/p17), capsid (CA/p24), nucleocapsid (NC/p7) and p6 with two spacer peptides, SP1, located between CA and NC, and SP2, found between NC and p6. These domains are separated by five flexible linker regions that contain sites that are cleaved by PR during virion maturation to liberate processed forms of the proteins. The Gag proteins direct virus particle production and perform critical roles in the early stages of viral replication.

The MA domain, found at the N-terminus of the Gag precursor, performs several essential functions during the virus lifecycle. Following synthesis, MA directs Gag to the plasma membrane through a multipartite membrane binding signal consisting of a myristate moiety covalently attached to the N-terminal glycine of MA and an N-proximal highly basic region (300). Mutational studies have demonstrated the importance of these regions (131, 247, 339) and demonstrate the ability of the highly basic region to interact with acidic phospholipids (344). It has been shown that this region interacts with phosphatidylinositol bisphosphate (PI(4,5)P2) (246), supporting a “mysistoyl switch” model for membrane targeting (246, 247, 251). In this model, interactions of the highly basic region with the negatively charged acidic phospholipids stabilize membrane interactions, causing a conformational change in MA that exposes the myristate moiety and induces membrane anchoring. In addition to directing Gag to the plasma membrane, MA has been shown to be important for Env incorporation during virus assembly, most

likely through interactions with the cytoplasmic tail of TM/gp120 (113, 115, 216). Following virus maturation, MA remains associated with the viral membrane, with some molecules present in the viral core where they were reported to contribute to viral core uncoating (176) and possibly PIC entry into the nucleus (40, 323).

C-terminal to MA in the Gag precursor is CA. CA is made up of two structural and functional domains, the N-terminal core domain and the C-terminal dimerization domain, that together enable CA to have several roles during viral replication. The C-terminal domain is primarily responsible for mediating Gag:Gag and Gag:Gag-Pol interactions necessary for virus particle and core assembly (86, 121, 158, 217) while the N-terminal core domain is needed for CypA incorporation, a protein thought to be critical for viral infectivity (110, 313). In the mature virion, CA is arranged into a hexameric and pentameric lattice that forms the outer shell of the viral core, protecting the inner viral ribonucleic particle (RNP) (123, 124).

The third domain found in Gag is NC, which has the primary function of encapsidation of the genomic RNA dimer. NC mediates gRNA incorporation through interactions with the two zinc-finger motifs of NC and the packaging signal (ψ) on the vRNA; a 150 nt sequence that folds into series of four stem loops. This interaction is also thought to act as a scaffold to mediate Gag nucleation and Gag multimerization (45, 79, 112, 236, 249, 283). NC remains tightly associated with gRNA in the mature particle and part of the viral core which has been demonstrated to act as a chaperone in early steps of virus replication by enhancing reverse transcription and integration (50, 199, 309). NC is flanked by the two spacer peptides which are cleaved to not only allow proper NC

functions but also virus assembly, maturation and infectivity. Specifically, the SP1 peptide is needed for immature particle assembly and regulating the formation of the mature capsid (3, 257), whereas the role of SP2 is less clear. Studies have suggested that while it *per se* may be dispensable for virus maturation and infectivity, processing of the NC-SP2-p6 region appears to be essential for proper viral morphology, maturation and infectivity (69, 82).

The p6 domain makes up the C-terminus of Gag and has two major functions. The first is to mediate Vpr incorporation into the assembling particles (254), the second is to promote detachment of assembled virus from the plasma membrane through motifs that recruit and bind to ESCRT (endosomal sorting complex required for transport) factors (300). The conserved Pro-Thr-Ala-Pro (PTAP) domain in p6 binds the Tsg101 subunit of the ESCRT-I complex (125, 321) while the Tyr-Pro-X_n-Leu (YPX_nL) motif interacts with the ESCRT protein ALIX (297). These interactions recruit other ESCRT proteins, namely ESCRT-III and VPS4 complexes, needed to complete particle release (300).

The other structural proteins are encoded within the *env* gene, and like Gag are synthesized as a polyprotein precursor. The 160 kD Env precursor is synthesized from a bicistronic singly spliced mRNA on the rough endoplasmic reticulum (ER) ribosomes where it is co-translationally glycosylated, inserted in the lumen of the ER and oligomerizes (114). Following trimerization, Env is transported to the Golgi where it undergoes further modification and is cleaved by cellular furin or furin-like proteases to the mature gp120 and gp41 proteins (144). Once cleaved, gp120 and gp41 non-

covalently associate and are transported from the Golgi to the plasma membrane where they are incorporated into the assembling virus particle (114). The primary function of the Env glycoproteins gp120 and gp41 is to mediate viral attachment and membrane fusion for viral entry into host cell. Viral entry traditionally has been thought to begin with binding of gp120 to cell surface receptor CD4. While this step is still necessary for viral entry limited evidence suggests that α4β7 integrins may also play a role in attachment and transmission in a subset of cell types (62). Once bound to the CD4 receptor, gp120 undergoes conformational changes that result in movement of the variable loops that then expose the gp120 co-receptor binding site and allow co-receptor binding, either CXCR4 or CCR5 (187, 332). Co-receptor binding of gp120 is thought to occur through its interactions of its bridging sheet and additional amino acids of the V3 loop (61). Co-receptor engagement induces additional rearrangements in gp120 and changes in gp41 that permits insertion of the gp41 fusion peptide, located at the N-terminus of the protein, into the host membrane and formation of the fusion pore (61). Next, three HR1 (heptad-repeat region 1) motifs make antiparallel interactions between three HR2 (heptad-repeat region 2) motifs to form a six-helix bundle that bring the viral and cellular membranes together, thus allowing for membrane fusion and entry of the HIV capsid into the target cell (61).

1.3.3 The Enzymatic Proteins

The *pol* gene encodes the enzymatic proteins protease (PR), reverse transcriptase (RT) and integrase (IN) and is found 3' (downstream) of *gag* in an overlapping but separate translational reading frame that lacks an initiation codon. As a result, *pol* is

synthesized as a 160 kD Gag-Pol fusion polyprotein precursor ($Pr160^{Gag\text{-}Pol}$, Gag-Pol) that is later proteolytically cleaved to produce the enzymatic proteins. Pol expression is dependent upon a the translational mechanism known as single (-1) ribosomal frameshifting (168), meaning ribosomes occasionally stall and shift reading frames. This mechanism is promoted by two RNA cis-acting elements found within the overlapping *pol* and *gag* genes, a UUUUUUA heptamer known as the "slippery sequence" and a frameshift A-form helical stem loop with an AACA tetraloop cap (150, 293). The exact mechanism of frameshifting has not yet been completely resolved, however, there is evidence that stem loop structure and stability are a contributing factor to its rate (24, 311), a frequency which occurs at a level of 5%-10% of Gag synthesis and is strictly maintained to ensure the critical 20:1 Gag:Gag-Pol ratio for viral replication (150). Once translated, Gag-Pol is targeted to sites of virus assembly at the plasma membrane, directed by MA, and packaged into the virion through interactions with CA. Either during or shortly after virus budding from the cell membrane, PR is autocatalytically released and dimerizes. PR then sequentially processes Gag and Gag-Pol to liberate the mature forms of their proteins, a necessary step for virion maturation and subsequent viral replication (150). The liberated RT enzyme is responsible for the conversion of the viral single-stranded RNA into double-stranded DNA during the early stage of replication known as reverse transcription. RT is able to catalyze this reaction through its three enzymatic functions: 1) RNA-directed DNA polymerase activity, which is necessary for the synthesis of the minus-strand DNA, 2) DNA-directed DNA polymerase activity, which is necessary for the synthesis of the plus-strand DNA and 3) RNaseH activity,

which is necessary for RNA degradation of RNA:DNA hybrids (279). RT does not have an exonucleolytic proofreading activity, meaning the enzyme is unable to recognize or rectify when an incorrect base pair is inserted into the DNA. This deficiency coupled with frequent template switching leads to high mutation rates, measured in cell-based assays at 3×10^{-5} per replication cycle, allowing for the advantage of virus genetic diversity (264, 312).

After reverse transcription, IN, the last enzyme found in Pol, binds to the dsDNA and mediates its insertion into the host genome to establish the provirus. IN catalyzes this reaction first in the cytoplasm where it binds to the linear dsDNA, processes the 3' ends by removing 3-4 nucleotides and generates the PIC. The PIC is then transported to the nucleus (see section 1.1.2 and Figure 1-2) where IN catalyzes the second reaction, known as the strand transfer reaction (STR). IN utilizes the 3' recessed ends of the viral DNA to attack the phosphodiester bonds in the major groove of cellular DNA which results in ligation of the ends in a 5-base pair stagger (220). Cellular repair machinery then trims and fills in the gaps of the integrated proviral DNA to complete the integration process.

1.3.4 The Accessory Proteins

There are four viral proteins categorized as the accessory proteins; Vif, Vpu, Vpr and Nef. The term "accessory" was assigned to these proteins because of early studies showing they were dispensable for viral replication in cultured cells (314). However, over the years the term "accessory" has been demonstrated to be quite a misnomer, as

each protein performs necessary functions for infectious particle production (116, 146, 238, 314).

Vif is a 23 kD protein produced during the late stage of the HIV-1 replication cycle and is required to counteract the restriction activity of the cellular protein apolipoprotein B messenger RNA (mRNA)-editing enzyme catalytic polypeptide-like 3G (A3G) (146, 211). A3G is a cytosine deaminase that is expressed in hematopoietic cells, a subset of which is a natural target for HIV-1 infection (211). In the absence of Vif, A3G is incorporated into nascent virions by binding to assembling Gag ribonucleoprotein (RNP) complexes (298). Thus, A3G is transferred to the target cell upon viral infection where it deaminates viral minus-strand cDNA, converting cytidines to uridines. These mutations cause the insertion of adenosines during positive-strand viral cDNA synthesis of reverse transcription. Remarkably, up to 10% of the viral cDNA cytidines are deaminated, which results in mutational changes in the viral genome, causing viral catastrophe (146, 211). Vif is able to successfully antagonize A3G activity by associating it with an ubiquitin ligase complex and induce its proteosome-dependent degradation (146, 211).

The HIV-1 viral accessory protein Vpu also functions to counteract host cell antiviral restriction activities. Vpu is a 16 kD integral membrane protein produced from a Rev-dependent bicistronic vRNA that also encodes the Env polyprotein and has at least two major functional roles during HIV-1 replication. First, Vpu induces the proteosomal degradation of the primary HIV-1 receptor CD4 molecules that are retained in the ER; a function most likely to prevent super-infection of the host cell as well as to counteract the

ability of CD4 to inhibit virus release and to interfere with the Env incorporation (8, 29, 146, 190). The second role for Vpu during viral replication is to regulate nascent particle release from the host cell surface (238). Vpu modulates particle release by antagonizing the cellular protein Tetherin, also known as B cell stromal factor 2 (BST2). In the absence of Vpu, Tetherin causes the accumulation of fully mature viral particles on the cell exterior. The mechanism of how Tetherin inhibits virus release has not been clearly defined. The prevailing model is that Tetherin physically tethers viral particles to the cell membrane as a dimer, interacting with both the cell and the virus (8, 146, 238). Also not clearly understood is the mechanism by which Vpu counteracts the antiviral activity of Tetherin; whether Vpu promotes the degradation, internalization or sequestration of the cellular protein continues to be investigated (8, 146, 238). Nonetheless, it is clear Vpu is required for enhanced particle release in the presence of Tetherin.

Nef is a membrane-associated, 27 kD phosphoprotein critical for HIV-1 viral infectivity and disease progression. There are three major functions that have been reported for Nef during viral replication: 1) down-regulation of CD4 and major histocompatability complex I (MHC I) expression from the host cell surface, 2) enhancement of virus infectivity, and 3) modulating T cell activation pathways (108, 116, 320). Nef mediates the down-regulation of CD4 and MHC I by triggering the rapid internalization and endosomal degradation of the proteins at the cell surface. The mechanism by which Nef enhances viral infectivity is not fully understood, however, has been proposed the small number of Nef proteins incorporated into nascent virions may promote viral core penetration of the target cell by inducing cortical actin rearrangements

as well as activating reverse transcription. Lastly, Nef has been shown to alter T cell activation pathways by modulating the activity of specific cellular kinases, a function suggested being important for viral persistence and pathogenesis.

The viral protein Vpr is incorporated at high levels into virus particles and has critical functional roles during viral infection (182). Although still not clearly defined, Vpr is thought to have an important role in the localization of the HIV-1 PIC to the nucleus by promoting the binding of the PIC to cellular importins and nucleoporins. Vpr nuclear localization has been proposed to have other functions, including stimulation of HIV-1 LTR driven transcription and, the most widely described, ability to arrest infected cells in the G_2 phase of the cell cycle (116, 182). Furthermore, Vpr has been implicated as the direct effector of immune dysfunction and neurological and renal AIDS associated diseases (182). However, the precise function of Vpr during viral replication remains to be determined.

1.3.5 The Regulatory Proteins

The HIV-1 genome encodes two regulatory proteins, Tat and Rev, both of which are essential for virus replication. The Tat protein, encoded by two exons in the *tat* gene, is necessary for the activation of RNA polymerase II (Pol II) transcription elongation of the viral RNA (116). In the absence of Tat, Pol II is unable to complete viral RNA synthesis; instead, it proceeds only for a couple hundred nucleotides (or less) before it falls off (116, 173, 174). Tat is able to activate successful elongation through its two functional domains, a tripartite activation domain and a RNA domain. The activation

domain includes a stretch of acidic amino acids at the N-terminus, a cystine-rich region and a hydrophobic core that are critical in the recruitment of the TAK (Tat-associated kinase, P-TEFb, CDK9/ cyclin T1 complex) complex to TAR element found in all vRNA transcripts (116, 173, 174). The RNA domain contains an 8 nt Lys/Arg-rich (nt 48-57) region that binds to the bulge region in TAR. Tat is recruited to the transcription complex and binds to the TAR in the viral RNA and forms a ternary complex with TAK, inducing activation of the TAK complex (116, 173, 174). Activated TAK phosphorylates the carboxy terminal domain (CTD) of Pol II, which improves processivity by several orders of magnitude.

The regulatory protein Rev is a 116 amino acid nuclear-cytoplasmic shuttling protein essential for virus replication. Rev contains two functional domains, an arginine rich motif (ARM) and an effector domain, both of which are necessary to mediate the main function of Rev, the nuclear export and expression of intron-containing vRNAs (reviewed in (47, 155, 260)). The ARM domain not only confers sequence-specific binding to the RRE, a stem-loop structure located within the intron-containing vRNAs, it also contains overlapping nuclear and nucleolar localization signals (NLS, NoLS) (66, 212, 214). The ARM domain is flanked by two oligomerization domains which mediate Rev multimerization, a requirement for Rev activity (93, 294, 342). The effector domain contains a leucine-rich nuclear export signal (NES) that interacts with the cellular proteins CRM1, Ran-GTP and other cofactors to facilitate export of intron-containing RNAs (13, 106, 174). In addition to its role in the export of intron-containing vRNAs, there is increasing evidence to support functional roles for Rev in the cytoplasmic

localization and translation of these vRNAs as well as in the enhancement of gRNA encapsidation (reviewed in (141)). Furthermore, Rev-dependent vRNA expression and regulation has been demonstrated to be tremendously complex, involving a growing list of host cellular factors (224, 330).

1.4 Post-transcriptional Regulation of HIV-1 Gene Expression

HIV-1 produces a primary transcript (pre-mRNA) from the integrated proviral genome that encodes all the necessary information to generate every viral component for infectious particle production (111, 116, 266). To ensure the necessary balance of vRNA expression from the pre-mRNA, HIV-1 exploits a variety of cellular mechanisms to orchestrate proper vRNA processing, export and utilization for infectious particle production. Thus, HIV-1 employs strategies that involve a dynamic interactome between vRNA, viral proteins and cellular co-factors that is initiated during vRNA transcription and continues through the cytoplasmic fate of the vRNA (139, 141, 174, 224, 330).

1.4.1 HIV-1 RNA Processing

Similar to cellular primary transcripts, HIV-1 pre-mRNA undergoes a series of modifications prior to their export, such as capping, 3’ end processing and polyadenylation. To carry out these post-transcriptional processes, HIV-1 usurps the host cellular machinery. Importantly, in order to produce the full range of vRNAs needed for viral protein expression and infectious virus production from pre-mRNA, HIV-1 must utilize alternative splicing mechanisms (111, 116, 266). The process of alternative splicing of both cellular mRNA and vRNA is quite complex and still being elucidated.

Nonetheless, a basic description is that the 5' splice donor site found within the pre-mRNA is recognized by the cellular splicing component U1 snRNP, while the 3' splice site is recognized by U2 snRNP in combination with the splicing factor U2AF65/35 to mediate excision of the intron. Most strains of HIV-1 pre-mRNA contain five splice donor sites and seven splice acceptor sites, thus providing variable usage, leading to the production of over 40 different transcripts during replication (Figure 1-4) (116, 167, 174, 224).

The production of such a vast number of alternative transcripts is attributed to the inefficient splicing of imperfect splice acceptor site (174, 224). In addition, the efficiency of the splice sites used during HIV-1 pre-mRNA splicing has been shown to be influenced by cellular factors binding to adjacent sequences (49, 174, 224). These regulatory sequences can either enhance or inhibit cellular factor binding and functions are known as exonic and intronic splicing enhancers (ESE, ISE) and exonic and intronic splicing silencers (ESS, ISS). Recent research has provided a more detailed understanding of which factors bind to these elements in HIV-1 pre-mRNA to regulate viral transcript production, however full understanding of the complexity of regulation is far from being realized (49, 174, 224). For example, Zahler *et al.* identified an overlapping ESE and an ESS located within tat exon 2 which was required for regulating the upstream intron. They demonstrated that heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) protein binds the ESS to repress splicing, while the SR protein SC35 binds the ESE to activate it, thus proposing that hnRNP A1 and SC35 compete for binding to the element such that binding of one protein masks the binding site for the other (341).

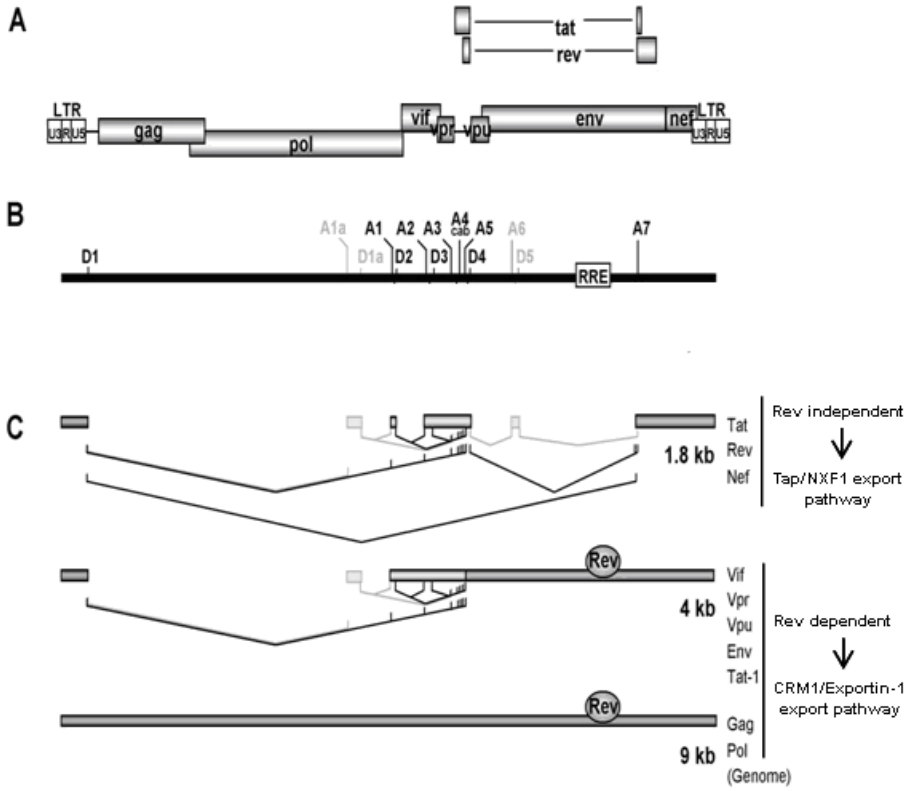

Figure 1-4. HIV-1 viral RNA splicing and export. (A) Organization of the HIV-1 genome. (B) Location of the splice sites and the Rev responsive element (RRE). 5' splice sites: D1a-5 and 3' splice sites: A1-7. (C) Splicing patterns, the proteins they encode and their mode of export. The 1.8 kb and 4 kb classes of vRNAs contain obligatory sequences (dark grey) and alternative sequences (light grey) due to different splice site usage. Completely spliced vRNA do not contain the RRE therefore their export is Rev-independent, through the Tap/NXF1 mRNA export pathway. Nuclear export of the partially spliced and full-length vRNA is dependent on Rev binding to the RRE (shown) and shuttling through the CRM1/Exportin1 pathway. *Adapted from Kammler, A. et. al. Retrovirology. (2006).*

As a result of alternative splicing, HIV-1 produces viral transcripts that contain one or all of its introns. However, transcripts that contain introns are typically retained in the nucleus for further processing or degraded (235). Thus, HIV-1 must overcomethis nuclear retention for intron-containing vRNAs for export and expression.

1.4.2 HIV-1 RNA Export

After transcript processing, vRNAs are exported for expression. As described in section 1.2 of this chapter, there are two export pathways for HIV-1 vRNA, a Rev-independent and Rev-dependent pathway (Figure 1-4). Viral transcripts that are fully spliced, which encode the regulatory proteins Tat, Rev, and Nef, are exported via the Tap/NXF1 pathway, typical for cellular mRNAs, are Rev-independent. However, vRNAs that contain introns must overcome cellular mechanisms preventing their transport. Although it is not fully understood, nuclear retention of intron-containing vRNAs has been attributed to partial spliceosome assembly and specific sequences intrinsic to these vRNAs, which are likely bound by certain cellular factors to restrict export (167, 224). HIV-1 overcomes this nuclear retention by the viral protein Rev, which functions to export the intron-containing vRNAs through the CRM1 export pathway, a pathway used for ribosome subunit, U snRNA and 5S rRNA export. This pathway for intron-containing vRNAs is termed Rev-dependent.

Although it well established that Rev mediates intron-containing vRNA export, it is less clear whether Rev also directly inhibits splicing of these vRNAs (56, 73, 139, 155, 213, 260). It has been shown that HIV-1 suboptimal splicing is necessary for Rev-

mediated export, several splicing factors bind to Rev and Rev function may require partial binding of splicing factors to intron-containing vRNAs, thus suggesting Rev may inhibit splicing (56, 179, 262). However, it has also been reported that the ratios of intron-containing vRNAs to completely spliced vRNAs in the nucleus were unchanged in the presence or absence of Rev (105, 215). Rather, investigators observed a lack of cytoplasmic intron-containing vRNAs, thus suggesting Rev does not alter splicing patterns but instead only participates in the export of the intron-containing vRNAs.

A simple model to describe Rev-mediated export is as follows: Rev binds to the RRE in a sequence-specific manner, multimerizes along the vRNA and this complex formation causes the NES of Rev to then be exposed. CRM1 and RanGTP (Ras-related nuclear protein, guanosine triphosphate) are recruited to the Rev-vRNP complex, which then interacts with nuclear pore proteins while trafficking through the nuclear pore to the cytoplasm. Once in the cytoplasm, RanBP1 (Ran binding protein 1) and RanGAP (Ran GTPase activating protein) convert RanGTP to RanGDP (Ras-related nuclear protein, guanosine diphosphate) causing vRNP export complex disassembly. After Rev releases its cargo, Importin β binds to the NLS of Rev and interacts with RanGDP to mediate the transport of Rev back into the nucleus through the nuclear pore (155, 260). Although the export of the intron-containing vRNAs is directed by Rev, it is not exclusive to Rev; Rev has been demonstrated to coordinate with other cellular factors to mediate vRNA export and its nuclear import (reviewed in (224)). RNA helicases DDX1 and DDX3 have been shown to associate with Rev and/or the vRNA (99, 273, 333, 336). DDX1 has been suggested to modulate Rev nuclear localization and promote Rev binding to the RRE, and

thus may be important for Rev-mediated export. DDX3 has been suggested to be central in Rev-vRNP complex release from the nuclear pore and mediate the cytoplasmic release of the vRNAs from the complex. Other factors, such as Sam68 (Src-associated substrate during mitosis of 68 kD) and hRIP (human Rev-interacting protein), have also been shown to have a functional role in Rev-mediated export, either for cytoplasmic transport or release from the perinuclear space, respectively (201, 231, 278, 292, 338). However, the mechanistic details and importance of the aforementioned proteins, and others identified, during Rev-mediated export is still being elucidated.

1.4.3 Rev-dependent vRNA Localization and Utilization

Increasing evidence suggests Rev-vRNP composition during RNA processing and export can dictate cytoplasmic utilization of the cognate vRNA which can be determined in the nucleus, at the nuclear pore or in the cytoplasm (2, 19, 58, 59, 60, 65, 71, 147, 206, 209, 223, 228, 232, 233, 292). The influence of vRNP composition for cytoplasmic localization and utilization has been particularly observed for full-length gRNA. The gRNA serves as an mRNA template for Gag and Pol polyprotein synthesis as well as the genome that is packaged into nascent virions. Despite these multiple roles, HIV-1 is not known to have distinct pools of gRNA to select transcripts for translation or packaging (42). It has been suggested that Sam68 may enhance 3’ end processing of unspliced vRNA and mark the gRNA in the nucleus to promote cytoplasmic utilization (71, 223). The RNA helicase DDX24 has been shown to promote gRNA packaging (209). Moreover, hnRNP A2 and Staufen have been demonstrated to associate with the genomic

RNP, promoting gRNA trafficking and packaging (2, 19, 58, 59, 60, 228).

In addition to cellular proteins, Rev has also been suggested to have a functional role in cytoplasmic utilization of vRNAs (139, 141). Several groups have shown Rev to enhance polysomal association of different intron-containing vRNAs, leading to their increased protein expression (11, 12, 76, 138, 141, 194, 256). Intriguingly, cells expressing cytoplasmic NLS-deficient Rev mutants were unable to promote intron-containing vRNA translation, thus indicating the translation enhancement function of Rev is determined in the nucleus (26, 237). Furthermore, nuclear Rev-vRNP composition has also been implicated to mediate Rev enhancement of gRNA encapsidation (26, 35, 134, 136). Überla *et al.* demonstrated that although a Rev-independent *gagpol* expression plasmid was efficiently expressed in the cytoplasm, it was inefficiently packaged; only when Rev was co-expressed with these transcripts was packaging enhanced (26, 35). However, the precise mechanism for Rev and vRNP components coordinating with Rev to promote gRNA packaging is presently unclear.

1.4.4 HIV-1 and the Nucleolus

The eukaryotic nucleolus is most notably recognized as the site for ribosomal RNA (rRNA) synthesis, processing and ribosomal subunit assembly. However, research over the past decade has demonstrated the nucleolus is multifunctional, involved in various aspects of cell biology, including cell stress sensing, cell cycle regulation and signal recognition particle biosynthesis (reviewed in (27, 245)). Several of the proteins that function at various aspects of HIV-1 post-transcriptional gene regulation described

herein have also been found to localize to the nucleolus The proteins that have been identified include both viral and cellular proteins (7, 77, 271, 282, 294, 333). As mentioned in section 1.3.5 above, the RNA binding domain of Rev also contains an overlapping NLS/NoLS, which mediates it predominate nucleolar localization (66, 155, 156). However, whether Rev nucleolar localization in necessary for function remains inconclusive.

It has been reported that Rev can induce the relocalization of CRM1 and Nup 98 and Nup214, nucleoporins (Nups) suggested to be important for Rev-directed RNA export, from the nucleus to the nucleolus, which suggests that the formation of the Rev export complex occurs in the nucleolus (346). Furthermore, studies indicate Rev can multimerize in the nucleolus and can actively bind and export a nucleolar targeted RRE when localized in this compartment, demonstrating Rev is functional while in the nucleolus (41, 70). The cellular proteins DDX1, hnRNP A1 have also been demonstrated to localize to the nucleolus (99, 192). Importantly, the nucleolus has been implicated to be the site for vRNA localization. Collectively, these results suggest a nucleolar localization step for vRNA during HIV-1 replication. More specifically, the nucleolus may have a functional involvement in Rev-directed RNA export.

1.4.4.1 HIV-1 Rev and Nucleophosmin/ NPM1

The nucleolar phosphoprotein Nucleophosmin (NPM1) is a ubiquitously expressed shuttling protein that has a role in numerous cellular activities regulated through the phosphorylation, acetylation, ubiquitylation, and SUMOylation of its

functional domains (117, 205, 241). NPM1 has been demonstrated to bind both DNA and RNA, promote processing of RNA, prevent protein misfolding and interact with several RNA processing factors (205, 241). NPM1 has also been identified to interact with HIV-1 Rev and has been suggested to be a Rev co-factor (100, 304, 305, 306). More specifically, several groups have shown Rev co-localizes with NPM1 in the nucleolus and *in vitro* analysis has demonstrated NPM1 directly interacts with Rev (91, 100, 151, 229, 230, 304, 306). Thus, it has been proposed NPM1 is the receptor mediating Rev nuclear import and/or nucleolar localization. However, its precise role in Rev function has yet to be identified.

1.5 Scope of Thesis

Although the nucleolus has been implicated to have a role during HIV-1 viral replication, its precise function has not been elucidated. In this thesis I present findings to support a functional involvement of the nucleolus during viral replication. My research addresses whether there is Rev-mediated nucleolar localization for intron-containing vRNAs prior to export. Moreover, I investigate whether NPM1 participates in Rev nucleolar localization and function and/or has a post-transcriptional role during viral replication. Since the viral protein Rev and the expression of intron-containing vRNAs are required for the production of infectious virus, establishing the functional involvement of the nucleolus during this process would not only enhance our current understanding of HIV-1 replication but could potentially provide new mechanistic insights, possibly leading to alternative therapeutic strategies.

CHAPTER II: MATERIALS AND METHODS

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2.1 Cell culture

2.1.1 HeLa, 293T, TZM-bl and HeLa-LAV cell line derivation and culture

HeLa, 293T (American Type Culture Collection, ATCC), TZM-bl and HeLa-LAV cell lines (obtained through the NIH AIDS Research & Reference Program, Division of AIDS, NIH: TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. and HeLa-LAV from Dr. Joerg Berg and Dr. Matthias Walb) The HeLa cell line is derived from human cervical epithelial cells (127). 293T cells are a derivative of the 293 human embryonic kidney epithelial cell line that carries the Simian Vacuolating Virus 40 (SV40) large T antigen. Expression of SV40T antigen allows for episomal replication of transfected plasmids containing the SV40 origin and the production of high titers of retrovirus (132, 255). TZM-bl cells are a HeLa-derived indicator cell line that can be used in quantitative analysis of HIV-1. These cells express the receptor CD4 and co-receptors CCR5 and CXCR4 on the cell surface and contain integrated copies of the luciferase and β-galactosidase reporter genes that are under the transcriptional control of the HIV-1 promotor (43, 259, 308, 328). HeLa-LAV cells are a HeLa-derived cell line that is stably infected with the HIV-1_{BRU} (LAV) strain, thus produce all viral proteins and release infectious virus particles (17, 18). Cells were maintained in 1X Delbecco Modified Eagle Medium (DMEM; Invitrogen) supplemented with 3.7 g/L sodium bicarbonate, 2.0 g/L HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 10% fetal bovine serum (FBS; BioWhittiker) and 0.5% Penicillin/Streptomycin (Pen/Strep; Invitrogen) at 37˚C and 5% carbon dioxide (CO_2).

2.1.2 Primary human monocyte-derived macrophages (MDM)

Elutriated primary human monocytes were obtained from the University of Massachusetts Medical School CFAR Core. Cells were differentiated into macrophages by culturing in 1.5 mL of 1X DMEM supplemented with 10% human AB male serum (SeraCare Life Sciences), 1% L-glutamine (Invitrogen) and 10 ng/mL of monocyte-colony stimulating factor (M-CSF; R&D Systems) in 6-well plates at a density of 1.25 x 10^6 cells per well for 4 days at 37˚C and 5% CO_2. On day 5, 1 mL of fresh media without M-CSF (referred to as macrophage media) was added to each well.

2.2 Plasmids

pCMV-βgal: a mammalian reporter plasmid expressing high levels of β-galactosidase, an internal control in experiments to monitor transfection efficiency. This reagent is commercially available from Clontech (cat. no. 631719).

pGFP-NPM1: a plasmid that expressed wild type, full length mammalian NPM1 fused to the enhanced green fluorescent protein (EGFP, GFP; a kind gift from Dr. Brunangelo Falini (97, 98)).

pGFP-NPM1MutA: a plasmid that expresses a mutant form of mammalian NPM1, a duplication of TCTG at positions 956-959 of the reference sequence. This frame shift causes amino acid changes (addition and replacements) at the C-terminal of the protein and results in a dominant-negative phenotype (a kind gift from Dr. Brunangelo Falini (97, 98)).

pBC12/CMV/IL-2: a plasmid that expresses secreted human interleukin-2 (IL-2) under the control of the cytomegalovirus (CMV) promotor and was used as a control. This reagent was a kind gift from Dr. Bryan R. Cullen (74).

pCX4pur: a retroviral cloning vector (Accession no. AB086386) carrying the puromycin N-acetyl-transferase (pac) gene from Streptomyces that confers cells expressing the vector resistant to puromycin. This reagent was a kind gift from Dr. Heinrich Gottlinger.

pcRev: a plasmid that expresses the viral protein Rev from the HXB3 strain of HIV-1 IIIB. This expression construct is derived from pBC12/CMV/IL2 in which IL-2 was excised from the *Hind* III and *Xho* I sites and replaced with Rev cDNA. This reagent was a kind gift from Dr. Bryan R. Cullen (214).

pHEF-VSV-G: a plasmid that expresses the Vesicular Stomatitis Virus Envelope Glycoprotein G (VSV-G) and was used to pseudotype HIV-1 particles. This reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. Lung-Ji Chang (57).

pMLVgagpol: a plasmid that expresses the Moloney murine leukemia virus Gag and Pol proteins. This reagent was a kind gift from Dr. Heinrich Gottlinger.

pNL4-3: a chimeric proviral clone that expresses full-length, replication and infection competent virus (NY5 and LAV HIV-1 strains). This reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. Malcolm Martin (4).

pNL4-3$_{48,71}$: a proviral clone derivative of pNL4-3 that contains two G:A point

mutations at positions 48 and 71 of the 234 nucleotide Rev-Response Element (RRE) which results in increased binding affinity of Rev to the RRE (16). This construct was generated from pNL4-3 using the QuikChange site-directed mutatgenesis kit (Stratagene) with oligonucleotides: (forward) 5'-CAGGAAGCACTATGGACGCAGCGTCAATGACGCTGACGATACAGGCCAGACAAT-3' and

(reverse) 5'-ATTGTCTGGCCTGTATCGTCAGCGTCATTGACGCTGCGTCCATAGTGCTTCCTG-3'.

The coding sequence of the resultant plasmid was validated by Sanger sequencing.

pNL4-3$_{\Delta env}$: a proviral clone derivative of pNL4-3 derivative containing two tandem premature stop codons in the Env reading frame and therefore lacks Env expression (a kind gift from Dr. Adonia L. Kim).

pNL4-3$_{\Delta Rev}$: a proviral clone derivative of pNL4-3 that contains a premature stop codon at amino acid 12 in the Rev open reading frame therefore prohibiting Rev expression, rendering the provirus replication incompetent (a kind gift from Dr. Marie-Louis Hammarskjöld (36)).

pRev-GFP: a plasmid that expresses the HIV-1 viral protein Rev fused to GFP. The Rev protein lacks the last six amino acids of the C-terminal and is followed by an Asp-Ala-Gln-Glu peptide linker and GFP (a kind gift from Dr. Barbara Felber (294)).

pTZ18U: an empty plasmid used as carrier DNA for transfections. This reagent was a kind gift from Dr. Heinrich Gottlinger.

2.3 Virus production

Viral stocks of VSV-G pseudotyped NL4-3, NL4-$3_{48,71}$, NL4-$3_{\Delta Rev}$ or NL4-$3_{\Delta env}$ were produced in 293T cells by transient transfection using the calcium phosphate method. Cells were seeded on 10 cm tissue culture dishes at 50-60% confluence in antibiotic-free 1X DMEM 24hrs prior to transfection. The following day, each transfection reaction was set up as follows: 10 μg of pHEF-VSV-G and 10 μg of either NL4-3, NL4-$3_{48,71}$, NL4-$3_{\Delta Rev}$ or NL4-$3_{\Delta env}$ was diluted in sterile water with the addition 124 μL of calcium phosphate up to total volume of 1 mL. 1 mL of 2X HEPES Buffered Saline [50 mM HEPES pH 7.05, 10 mM KCL, 12 mM Glucose, 250 mM NaCl, 1.5 mM Na_2HPO_4] was added drop-wise to the DNA mixture as it was being vortexed, and incubated at room temperature for 10 minutes. Culture media was replaced with 8 mL of fresh antibiotic-free 1X DMEM supplemented with 4 μL of 25 mM chloroquine per plate before the drop-wise addition of the transfection reaction then cells were maintained at 37˚C, 5% CO_2. After 14-to 16hrs, cells were washed twice with 1X Phosphate Buffered Saline (PBS) and fresh antibiotic-free 1X DMEM was added. Culture supernatants were collected at 48hrs post-transfection, clarified by centrifugation at 1200 rpm for 5 minutes at room temperature, then aliquoted and stored in liquid nitrogen until used.

2.4 Virus titer and infectivity assay

2.4.1 Virus titer

Virus stock titers were determined as follows: TZM-bl cells were seeded in 48-well dishes at a density of 2.0 x 10^4 cells per well in antibiotic-free 1X DMEM one day prior to infection. The next day, virus stock was thawed at 37˚C. 300 μL was transferred to a sterile tube to start three subsequent 10-fold serial dilutions made up to a total volume of 300 μL in serum-free media. Cell monolayers were inoculated in duplicate with 100 μL of serum-free 1X DMEM, virus or diluted virus for 3hrs prior to the addition of 400 μL of serum-free 1X DMEM to each well. At 48hrs post infection, culture media was removed, cells washed twice in 1X PBS and fixed with 0.5% gluteraldehyde in 1X PBS for 10 minutes at 4˚C. Fix solution was then removed and cells were washed twice in 1X PBS/0.05% sodium azide and stored in solution until assayed for β-galactosidase (β-gal) activity. To determine virus titer, TZM-bl cells were stained for 2hrs at room temperature with 200 μL of 0.5 mg/mL 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranocide diluted in a 1X PBS, 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, 1 mM magnesium chloride solution. Viral titer (Infectious Units per μL) for each stock was determined by counting the number of β-gal expressing cells (blue cells) in duplicate wells.

2.4.2 Virus infectivity assay

The infectivity of virus produced from siRNA treated and/or infected HeLa cells (Chapter IV) was determined using the same protocol detailed above for determining virus titer with one exception: instead of undiluted virus, equivalent reverse-transcriptase

activity units in a total volume of 300 μL were used for infection and to prepare the three subsequent 10-fold serial dilutions.

2.5 Virus infection

Prior to infection, HeLa, TZM-bl or MDM cells had culture media removed and were washed 3 times in 1X PBS. Cells were either uninfected (media only) or infected with VSV-G pseudotyped virus diluted in antibiotic-free 1X DMEM. Cells were infected at a multiplicity of infection (MOI) 1 or 4, as determined by section 2.4, for 4hrs at 37˚C, 5% CO_2. At 4hrs post infection, culture media was removed, cells were washed 3 times in 1X PBS and fresh antibiotic-free 1X DMEM was added. Cells were maintained at 37˚C, 5% CO_2 for the duration of the experiments.

2.6 Reverse transcriptase (RT) activity assay

Culture supernatants were lysed with 0.5% Triton X100 in 0.25 M Tris, pH 7.4 and assayed in triplicate for the quantification of reverse transcription activity. For each reaction, 10 μL supernatant and 10 μL of solution A [100 mM Tris-HCL pH 7.9, 300 mM potassium chloride, 10 nM dithiothreitol, 0.1% Igepal CA-630] was mixed in a 96-well round bottom plate and incubated at 37˚C for 15 minutes. Next, 25 μL of solution B [50 mM Tris-HCL pH 7.9, 150 mM potassium chloride, 5 mM dithiothreitol, 15 mM magnesium chloride, 0.5% Igepal CA-630, 10 μg/mL polyadenylic acid, 5 μg/mL oligo-dT_{15}, 10 μCi [^{3}H]-dTTP/mL] was added, mixed and incubated overnight at 37˚C. Reactions were blotted onto 2.3 cm DE81 Whatman ion exchange filter disks, air dried,

washed twice in 2X SSC [0.3 M sodium chloride, 0.03 M sodium citrate] on a shaker table for 5 minutes and rinsed briefly in 100% ethanol. Air dried filter disks were placed into scintillation vials and 5 mL of Ecoscint scintillation fluid (National Diagnostics) was added. A Beckman Coulter LS6500 Scintillation Counter was used to detect and measure tritium counts per minute (cpm) for 2.5 minutes per vial. To calculate percent virus production, the mean cpm for each infection condition within each experiment was first determined and subtracted from the mean cpm of each corresponding sample lacking virus. The adjusted cpm mean was normalized to the Mock condition within the experiment to obtain the resulting percentage. Data graphs represent the total mean percent virus production from the indicated number of experiments in the figure legends ± Standard Error of the Mean (SEM). Multiple student T-test was used for statistical analysis (GraphPad Prism6).

2.7 Western blot analysis

2.7.1 HeLa, TZM-bl, HeLa LAV and HeLa-derived stable cell lines

Culture media was removed by aspiration, cells were washed 3 times in 1X PBS, then a volume of 1X PBS was added to each culture vessel: 500 μL per well of a 6-well plate or 1mL per 60 mm^2 and 10 cm^2 dish. Adherent cells were removed using a plastic cell scraper and collected by pipette into a 1.5 mL microfuge tube. 500 μL more of 1X PBS was added to each culture vessel and collected into the microfuge tube. Cells were maintained on ice. Next, cells were pelleted by centrifugation at 3000 rpm for 3 minutes at room temperature, supernatants were removed by aspiration, and whole cell lysates

were prepared by re-suspending cells in either lysis buffer [0.5% Triton X100, 0.25 M Tris, pH 7.4] or 1X RIPA buffer [100 mM Tris-HCL, pH 7.4, 300 mM NaCL, 2% NP-40, 1% Sodium deoxycholate, 0.2% SDS (Boston BioProducts)] and incubating on ice for 15 to 30 minutes. Solubilized proteins were clarified by centrifugation at 14000 rpm and supernatants were transferred to new tubes. Total protein concentration of cell lysates were determined using colormetric Bradford assays (BioRad). A total protein concentration of 12.5 μg of each lysate (unless otherwise noted in a specific material and methods subsection) was resolved on a 12% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (BioRad) using a semi-dry blotter. Membranes were subsequently blocked in Blotto (10% dry milk in 1X TBST buffer [25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4, and 0.1% Tween-20]) at room temperature for 1 hour, then incubated with primary antibody diluted in Blotto overnight at 4˚C. Primary antibodies used include: mouse monoclonal antibodies directed against NPM1 (Sigma, cat. no. B0556, diluted 1:10,000), HIV-1 Gag (NIH AIDS Research and Reference Reagent Program, cat. no. 183-H12-5C, diluted 1:500), HIV-1 Nef (NIH AIDS Research and Reference Reagent Program, cat. no. 1539, diluted 1:1000), GFP (Roche, cat. no 11814460001, diluted 1:500), FLAG (Sigma, cat. no. F4042) and rabbit or goat polyclonal antibodies directed against Sam68 (Santa Cruz Biotechnology, cat. no. sc-333, diluted 1:1000), human Actin (Santa Cruz Biotechnology, cat no. sc-1616, diluted 1:500), and hRIP (Santa Cruz Biotechnology, cat. no. sc-1424, dilution 1:500). Following three 5 minute washes in 1X TBST buffer, membranes were incubated with sheep anti-mouse (GE Healthcare, cat. no. NA931), donkey anti-rabbit (GE Healthcare, cat. no. NA934), or

donkey anti-goat (Santa Cruz Biotechnology, cat. no. SC2056) secondary antibodies conjugated to horseradish peroxidase (diluted 1:5000) for 1hr at room temperature. Membranes were washed 3 times for 5 minutes in 1X TBST and protein expression was visualized using enhanced chemiluminescence (Amersham Biosciences). Multiple protein expression profiles were detected from a single membrane by a strip and reprobe process. Briefly, membranes were washed 3 times in 1X TBST, incubated with Restore Stripping Buffer (Thermo Scientific) for 10 minutes at room temperature, followed by 3 washes in 1X TBST, then Western blotting proceeded as described.

2.7.2 MDM

Western blotting of MDM lysates followed the same protocol detailed above with the following exceptions: culture media and 1X PBS were manually removed by serological pipette instead of aspiration, cells were lysed directly in the culture vessels with lysis buffer [0.5% Triton X100, 0.25 M Tris, pH 7.4] rather than by cell scraping and lysates were not clarified by centrifugation.

2.8 siRNA transfections

2.8.1 HeLa, TZM -bl, HeLa-LAV and HeLa-derived stable cell lines

Cells were seeded on 6-well plates at a density of 7.25×10^4 cells per well (unless otherwise noted under a specific material and methods subsection) in antibiotic-free 1X DMEM 24hrs prior to siRNA transfection. Cells were transfected using Oligofectamine reagent (Invitrogen) according to manufacturer's protocol for 6-well culture dishes, with

reagent alone (Mock), 5 nM of Allstars Negative Control siRNA, a validated control siRNA duplex with no known homology to any mammalian gene (siANC; Qiagen cat. no. 1027281), or 5 nM of human nucleophosmin 1 (NPM1)-specific siRNA duplexes (siNPM1; Qiagen) at time 0-and 24hrs. Treatment with siNPM1 is an equimolar mixture of 2.5 nM of two validated NPM1-specific siRNA duplexes with the following sequences: Hs_ NPM1_7 siRNA: sense strand 5'-AGGUGGUUCUCUUCCCAAATT-3' and antisense strand 5'-UUUGGGAAGAGAACCACCUTT-3'. Hs_NPM1_8 siRNA: sense strand 5'-UGUCUGUACAGCCAACGGUTT-3' and antisense strand 5'-ACCGUUGGCUGUACAGACATT-3'. An alternative control siRNA used was 5 nM of siScr (Qiagen). Treatment with siScr is an equimolar 2.5 nM mixture of two validated scrambled sequences analogous to the Hs_ NPM1_7 and Hs_NPM1_8 target siRNAs with no known homology to any mammalian gene and the following sequences: NPM_7Scr: sense strand 5'-UGAACACUUAAGGUGCCAUTT-3' and antisense strand 5'-AUGGCACCUUAAGUGUUCAAG-3'. NPM1_8Scr: sense strand 5'-UAUGUAACUAACCGUGGCATT-3' and antisense strand 5'-UGCCACGGUUAGUUACAUAGC-3'. Cells were maintained at 37˚C, 5% CO_2 for the duration of the experiment.

2.8.2 MDM

Primary human monocytes were differentiated to MDM as described above in section 2.1. On day 6, cell culture media was collected in a 50 mL conical tube and diluted in half with fresh macrophage media [1X DMEM, 10% human AB male serum,

1% L-glutamine] to make a 50/50 conditioned media; meanwhile, cells were washed once with 1X PBS using a serological pipette and subsequently Mock transfected or transfected with either 10 nM of siANC or 10 nM siNPM1 using Lipofectamine 2000 reagent (Invitrogen) and a modified protocol. Briefly, for each siRNA reaction 10 nM of siRNA was diluted in Opti-MEM I media (Invitrogen) to a total volume of 500 μL in a 1.5 mL microfuge tube. In a separate tube for each reaction, 5 μL of Lipofectamine 2000 was diluted in 495 μL of Opti-MEM I media and incubated at room temperature for 5 minutes. Next, the diluted Lipofectamine 2000 was combined with the diluted siRNA reactions, mixed, and incubated at room temperature for 20 minutes. Each 1 mL reaction mixture was then added directly to cells and incubated at 37˚C, 5% CO_2 for 4hrs. The transfection reactions were subsequently removed from cells using a serological pipette, washed once with 1X PBS, and 1.5 mL of 50/50 conditioned media was added. Cells were maintained at 37˚C, 5% CO_2 for the duration of the experiment.

2.9 *In situ* hybridization and indirect immunofluorescence microscopy

Several experiments described in this thesis had slight method variations for *in situ* hybridization and indirect immunofluorescence microscopy analysis and are described below. For all experiments, cells were visualized using a Zeiss Axioplan 2 wide-field fluorescence microscope equipped with a 63x Plan Apochromat objective and standard fluorescence filters. Images were captured and analyzed using Open Lab 2.2.5 software.

2.9.1 Chapter III: Rev localization in HeLa LAV and provirus transfected HeLa cells

Cells were seeded on 10 cm^2 culture dishes containing 4 cover slips at a density of 7 x 10^5 cells per plate in antibiotic-free 1X DMEM 24hrs prior to transfection. HeLa LAV cells remained untreated. While HeLa cells were either transfected with reagent alone (Mock) or 3 μg of either pNL4-3, pNL4-$3_{\Delta Rev}$ or pNL4-$3_{48,71}$ and 1.5 μg of pCMVβ-gal using FugeneHD (Roche) transfection reagent according to the manufacturer's protocol at the 3:2 FugeneHD to DNA ratio. 24hrs later, culture supernatants were collected and assayed for RT activity as described in section 2.6, cells were collected, lysed and analyzed by Western blotting as described in section 2.7 and cover slips were placed into 6-well culture dishes, washed 3 times in 1X PBS, then fixed with 2 mL of ice cold 100% methanol for 5 minutes. Following fixation, cells were washed 3 times with 1X PBS, twice with 70% ethanol, and stored at 4˚C in 70% ethanol until stained. Next, cover slips were washed 3 times in 1X PBS for 10 minutes/wash, incubated with 1% Bovine Serum Albumin (BSA) in 1X PBS for 1hr, then incubated with an anti-Rev mouse monoclonal primary antibody (Santa Cruz cat. no. sc-69729) diluted at 1:200 in 1% BSA/1X PBS for 1.5hrs. Cells were subsequently washed 3 times in 0.1% BSA/1X PBS for 10 minutes/wash, and incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (Life Technologies, cat. no. A11001) diluted in 1% BSA/1X PBS at 1:1500 for 1hr, covered in foil. Following antibody incubation, cover slips were washed twice in 1X PBS for 5 minutes and incubated with DAPI [4', 6-DiamidinO-2-phenylindole] at a dilution of 1:10,000 from a 10 mg/mL stock solution in 1X PBS for 5 minutes. Excess DAPI was removed by washing twice in 1 X PBS for 5

minutes followed by a wash in double distilled water (ddH_20) for 20 minutes. All steps described were carried out at room temperature while rocking on a shaker table. Finally, cover slips were mounted onto slides with Prolong Gold Antifade Reagent (Invitrogen, cat. no. P36930) and sealed with clear nail lacquer. Slides were dried overnight in the dark and stored in a slide box at 4˚C until used.

2.9.2 Chapter III: Rev-dependent viral RNA (vRNA) localization

HeLa cells were seeded on 10 cm^2 culture dishes containing 4 cover slips at a density of 7 x 10^5 cells per plate in antibiotic-free 1X DMEM 24hrs prior to infection. Cells were infected with VSV-G pseudotyped NL4-3, NL4-$3_{\Delta Rev}$ or NL4-$3_{48,71}$ virus as described in section 2.5 at a MOI of 1. At 6-, 12-, 24-, or 30hrs culture supernatants were collected and assayed for RT activity as described in section 2.6. Cells were collected, lysed and analyzed by Western blotting as described in section 2.7. And cover slips were placed into 6-well culture dishes, washed 3 times in 1X PBS, then fixed [2% paraformaldehyde, 1 mM Magnesium Chloride, 1X PBS] for 15 minutes at room temperature, washed 3 times with 70% ethanol and stored in 70% ethanol at 4˚C until stained. Cells on cover slips were first rehydrated by washing twice in 1X PBS and then pretreated in hybridization solution [0.2X SSC, 1 mg/ml BSA, 30% deionized formamide, 10% dextran sulfate, 0.1 mg/mL herring sperm DNA, 0.1 mg/mL tRNA] sealed with parafilm for 1hr at 37˚C. Cover slips were further incubated in the dark with hybridization solution containing 100-150 ng of Cy3-conjugated (Amersham) RRE-specific oligonucleotide probe complimentary to nucleotides 7796-7872 of the HIV-1

NL4-3 strain (Accession no. M19921) or Cy3-conjugated *gag-pol*-specific oligonucleotide probe complimentary to nucleotides 2462-2553 of the HIV-1 NL4-3 strain, sealed with parafilm for 1hr at 37˚C. Excess hybridization solution was removed and cover slips were placed into 6-well culture dishes where they were subsequently incubated twice for 30 minutes in hybridization solution in the dark at 37˚C. Cover slips were then washed once in 2X SSC, 20% deionized formamide for 30 minutes followed by one wash in 1X SSC, 20% deionized formamide, two washes in 1X SSC and one wash in 1X PBS, for 15 minutes each at room temperature. Cells were blocked in 1% BSA/1X PBS overnight at 4˚C with rocking. The next day, cells were incubated in primary antibody, either anti-fibrillarin (abcam, cat. no. 18380, dilution 1:500) or anti-NPM1 (dilution 1:500), diluted in 1% BSA/1X PBS for 1hr in the dark at room temperature. Cover slips were then washed 3 times in 0.1% BSA/1X PBS for 10 minutes per wash and incubated in Alexa Fluor 488 conjugated goat anti-mouse secondary antibody at a 1:1500 dilution in 1% BSA/1 XPBS for 1hr, at room temperature. Following antibody incubation, cover slips were washed twice in 1X PBS for 5 minutes and incubated with DAPI at a dilution of 1:10,000 from a 10 mg/mL stock solution in 1X PBS for 5 minutes. Excess DAPI was removed by washing twice in 1 X PBS for 5 minutes followed by a wash in dd$H_2$0 for 20 minutes. Lastly, cover slips were mounted onto slides with Prolong Gold Antifade Reagent and sealed with clear nail lacquer. Slides were dried overnight in the dark and stored in a slide box at 4˚C until used.

2.9.3 Chapter III: Visualizing NPM1 depletion in HeLa cells

HeLa cells were seeded on 60 mm^2 culture dishes at a density of 16.5 x 10^4 cells per plate in antibiotic-free 1X DMEM 24hrs prior to siRNA transfection. Cells were either untreated or transfected using Oligofectamine reagent according to the manufacturer's protocol with reagent alone (Mock) or 5 nM of siNPM1 at time 0 and 24hrs. At 24hrs post siRNA treatment, cells were washed 3 times with 1X PBS, trypsinized, and collected in 8 mL of antibiotic-free 1X DMEM for re-seeding. A volume of 1 mL of cells were re-seeded in triplicate onto cover slips in 6-well plates and brought to a final volume of 3 mL with media to be analyzed by indirect immunofluorescence microscopy. A volume of 4 mL of cells were re-seeded onto 60 mm^2 culture dishes to be analyzed by Western blotting. The next day, cells were washed 3 times in 1X PBS. Cells collected for Western blotting were harvested and processed as described in section 2.7 (a total protein concentration of 25 μg per lysate per analysis). Cover slips were fixed [4% paraformaldehyde, 1 mM Magnesium Chloride, 1X PBS] for 15 minutes at room temperature, washed 3 times with 70% ethanol and stored in 70% ethanol at 4˚C until used. Cover slips were washed twice in 1X PBS for 20 minutes, permeabolized [0.1% Triton X-100, 1X PBS] for 5 minutes, washed twice in 1X PBS for 5 minutes and finally incubated with 1% BSA/1X PBS overnight at 4˚C. The next day, cover slips were incubated with a mouse monoclonal anti-NPM1 primary antibody diluted in 1% BSA/1X PBS at 1:500 for 2hrs. Cover slips were subsequently washed 3 times in 0.1% BSA/1X PBS for 10 minutes and incubated with Alexa Fluor 488 goat anti-mouse secondary antibody diluted in 1% BSA/1X PBS at 1:1500 for 1hr. Following

antibody incubation, cover slips were washed twice in 1X PBS for 5 minutes and incubated with DAPI at a dilution of 1:10,000 from a 10 mg/mL stock solution in 1X PBS for 5 minutes. Excess DAPI was removed by washing twice in 1 X PBS for 5 minutes followed by a wash in ddH_20 for 20 minutes. All steps described were carried out at room temperature with rocking. Lastly, cover slips were mounted onto slides with Prolong Gold Antifade Reagent and sealed with clear nail lacquer. Slides were dried overnight in the dark and stored in a slide box at 4˚C until used.

2.9.4 Chapter III: Effect of NPM1 depletion on Rev intracellular localization

The same protocol detailed above in *Visualizing NPM1 depletion in HeLa cells* was used to analyze the effect of NPM1 depletion on Rev intracellular localization with the following exceptions: at 10hrs after the second siRNA transfection cells were washed 3 times in 1X PBS, trysinized, and 8.85 x 10^4 cells were re-seeded in triplicate onto cover slips in 6-well plates while 8.85 x 10^4 cells were re-seeded in duplicate on 6-well plates without cover slips for analysis by Western blotting. The day after re-seeding, cells were washed 3 times in 1X PBS, fed 3 mL of fresh antibiotic-free 1X DMEM, then transfected with 1 μg of pBC12/CMV/IL-2 (Mock) or 1 μg pRev-GFP for each siRNA treatment condition using FugeneHD reagent according to the manufacturer's protocol at the 3:2 FugeneHD to DNA ratio. Cover slips were fixed and cells were harvested for indirect immunofluorescence analysis and Western blotting, respectively, at 24-and 48hrs post-DNA transfection.

2.9.5 Chapter IV: Effect of NPM1 depletion on vRNA intracellular localization

HeLa cells were seeded on 60 mm^2 culture dishes at a density of 16.5 x 10^4 cells per plate in antibiotic-free 1X DMEM 24hrs prior to siRNA transfection. The next day, cells were transfected with siRNA as described in section 2.8. At 10hrs past the second siRNA transfection, cells were washed 3 times in 1X PBS, trysinized, and 8.85 x 10^4 cells were re-seeded in triplicate onto cover slips in 6-well plates while the remaining cells were re-seeded onto 60 mm^2 culture dishes without cover slips for analysis by Western blotting. The day after re-seeding, cells were washed 3 times in 1X PBS, fed 3 mL of fresh antibiotic-free 1X DMEM, then were either uninfected or infected with VSV-G pseudotyped NL4-3 virus at an MOI of 4 as described in section 2.5. At 24hrs post infection, culture supernatants were collected and assayed for RT activity as described in section 2.6, cells were collected, lysed and analyzed by Western blotting as described in section 2.7. Cover slips were washed 3 times in 1X PBS, then fixed [4% paraformaldehyde, 1 mM Magnesium Chloride, 1X PBS] for 15 minutes at room temperature, washed 3 times with 70% ethanol and stored in 70% ethanol at 4˚C until used. Cells on cover slips were rehydrated by washing twice in 1X PBS, then pretreated in hybridization solution [0.2X SSC, 1 mg/ml BSA, 30% deionized formamide, 10% dextran sulfate, 0.1 mg/mL herring sperm DNA, 0.1 mg/mL tRNA for cells to hybridize with an RRE-specific, gag-pol-specific probes; 40% deionized formaldehyde was used for hybridization with *env*-specific and GAPDH-specific probes] sealed with parafilm for 1hr at 37˚C. Cover slips were incubated in the dark with hybridization solution containing 100-150 ng of a Cy3-conjugated RRE-specific oligonucleotide probe, a Cy3-

conjugated *gag-pol*-specific oligonucleotide probe, a Cy3-conjugated *env*-specific oligonucleotide complimentary to nucleotides 6432-6503 of the HIV-1 NL4-3 strain, or Cy3-conjugated human glyceraldehyde-3-phoshate dehydrogenase (GAPDH)-specific oligonucleotide complimentary to nucleotides 121-179 of the mRNA (Accession no. M17851) and sealed with parafilm for 1hr at 37˚C. Excess hybridization solution was removed and cover slips were placed into 6-well culture dishes and incubated for 60 minutes in hybridization solution in the dark at 37˚C. Cover slips were then washed once in 2X SSC, 20% deionized formamide for 30 minutes followed by one wash in 1X SSC, 20% deionized formamide, two washes in 1X SSC and one wash in 1X PBS, for 15 minutes each at room temperature. Cells were blocked in 1% BSA/1X PBS overnight at 4˚C with rocking. The next day, cells were incubated in anti-NPM1 primary antibody diluted 1:500 in 1% BSA/1X PBS for 3hrs in the dark at room temperature. Cover slips were washed 3 times in 0.1% BSA/1X PBS for 10 minutes per wash and incubated in Alexa Fluor 488 conjugated goat anti-mouse secondary antibody at a 1:1500 dilution in 1% BSA/1 XPBS for 1hr at room temperature. Following antibody incubation, cover slips were washed twice in 1X PBS for 5 minutes and incubated with DAPI at a dilution of 1:10,000 from a 10 mg/mL stock solution in 1X PBS for 5 minutes. Excess DAPI was removed by washing twice in 1 X PBS for 5 minutes followed by a wash in ddH_20 for 20 minutes. Lastly, cover slips were mounted onto slides with Prolong Gold Antifade Reagent and sealed with clear nail lacquer. Slides were dried overnight in the dark and stored in a slide box at 4˚C until used.

2.10 Chloramphenicol aceytltransferase (CAT) activity assays

HeLa cells were seeded on 6-well plates at a density of 7.25 x 10^4 cells per well in antibiotic-free 1X DMEM 24hrs prior to siRNA transfection. Cells were transfected using Oligofectamine reagent according to manufacturer's protocol with reagent alone (Mock), 5 nM of siANC, or 5 nM siNPM1 at time 0hrs and 24hrs. At 24hrs post siRNA treatment, cells were washed 3 times in 1X PBS, fed 3 mL of fresh antibiotic-free 1X DMEM, then transfected with 2 μg of plasmid DNA using FugeneHD transfection reagent according to the manufacturer's protocol at the 3:2 FugeneHD to DNA ratio. Cells were transfected with 2 μg of pBC12/CMV/IL-2 (Mock), 1 μg of pCMV128 and 1 μg pBC12/CMV/IL-2, or 1 μg of pCMV128 and 1 μg pcRev in the absence or presence of each siRNA. At 24-or 48-hrs post-transfection, cells were washed with 1X PBS, scraped and collected in 1 mL of 1X PBS into microfuge tubes. Cells were pelleted by centrifugation at 3000 rpm for 3 minutes at room temperature, supernatants were removed, lysed in 100 μL of lysis buffer (0.5% Triton X-100 in 0.25 M Tris pH 7.4) and incubated on ice for 15 minutes. Supernatants were separated from cellular debris by centrifugation at 14,000 rpm for 10 minutes at 4˚C and transferred to new microfuge tubes. Total protein concentration of cell lysates was determined using colormetric Bradford assays. A total protein concentration of 12.5 μg of each lysate was resolved on a 12% SDS-PAGE gel, transferred to a nitrocellulose membrane, then analyzed with anti-NPM1 and anti-Actin antibodies as described in section 2.7. A total protein concentration of 15 μg of each lysate was aliquoted to new tubes, volume adjusted to 30 μL with lysis buffer then assayed for CAT enzyme activity. The assay mixture

containing 100μL of 0.25 M Tris pH 7.4, 0.1 μCi of [^{14}C] Chloramphenicol, 30 μL of 5 mg/mL Acetyl Coenzyme A, 4 μL of 80% glycerol, 20 μL of water and 30 μL of lysate, was incubated at 37˚C for 90 minutes. Reactions were stopped by the addition of 400 μL of ethyl acetate, vortexed, and centrifugated for 2 minutes at 14,000 rpm at room temperature. The organic layer was removed, air dried to a pellet, resuspended in 10 μL of ethyl acetate, spotted on a silica gel thin-layer plates and assay products were resolved using thin layer chromatography and autoradiography (342). To calculate the mean percent acetylation of each sample, the total percent of conversion of each point was first determined and then subtracted from the total percent acetylation of each corresponding sample lacking Rev using densitometric analysis using Image J software. A randomized block one-way ANOVA was used for statistical analysis at each time point (GraphPad Prism6).

2.11 p24 enzyme-linked immunosorbent assay (ELISA)

The quantification of HIV-1 p24 protein present in MDM culture supernatants obtained 24-and 48hrs post infection, was performed using a p24 ELISA kit (UMMS CFAR Core) according to the provided protocol. Culture supernatants were diluted in macrophage media [1X DMEM supplemented with 10% human AB male serum, 1% L-glutamine] to ensure measurement within the linear range of the assay. For each 96-well plate assay, a four-point calibration curve was prepared in duplicate from the provided p24 standard while two dilutions of each sample were assayed in duplicate. Protein concentration of the 96-well pate was determined by measuring the absorbance of each

well at 450/570 nm using a Bio-Tek EL800 microplatereader. The measured values were calculated and analyzed with KC Junior software (Bio-Tek Instruments). To calculate percent p24 production, the mean value for each condition was determined and normalized to the Mock condition within the experiment to obtain the corresponding percentage. Data graphs represent the total mean percent p24 production from the indicated number of experiments indicated in the figure legends ± SEM. Multiple student T-test was used for statistical analysis (GraphPad Prism6).

2.12 Development of NPM1 siRNA-resistant expressing stable cell lines

2.12.1 Generation of GFP-NPM1 siRNA resistant plasmids

Wild type pGFP-NPM1 was used to generate the pGFP-NPM1 siRNA-resistant clones. Silent mutations were introduced using the QuikChange site-directed mutagenesis kit according to the manufacturer's protocol, and the following primers: NPM7siR_2 (forward) 5'-GTATAGAAAAAGGTGGCAGCTTGCCCAAAGTGGAAGCC-3' and (reverse) 5'-GGCTTCCACTTTGGGCAAGCTGCCACCTTTTTCTATAC; NPM7siR_6 (forward) 5'- GCAAGTATAGAAAAAGGCGGCAGCTTGCCTAAGTGGAAGCCAAATTC-3' and (reverse) 5'-GAATTTGGCTTCCACTTAGGCAAGCTGCCGCCTTTTTCTATACTTGC-3'; NPM18siR_4 (forward) 5'-GCAACTTTGAAAATGAGCGTGCAACCAACGGTTTCCC-3' and (reverse) 5'-GGGAAACCGTTGGTTGCACGCTCATTTTCAAAGTTGC-3'; NPM18siR_8

(forward) 5'-GCAACTTTGAAAATGAGCGTGCAACCCACGGTTTCCCTTGG-3' and (reverse) 5'-CCAAGGGAAACCGTGGGTTGCACGCTCATTTTCAAAGTTGC-3'. The mutations within each expression construct were validated by Sanger sequencing. A diagram of the GFP-NPM1 siRNA-resistant clones and the silent mutations is shown in Figure 2-1. The GFP-NPM1 siRNA-resistant plasmids were cloned into the pCX4pur expression vector via the *EcoRI* and *NotI* restriction enzyme sites. The *EcoRI* and *NotI* restriction enzyme sites were introduced into GFP-NPM1 siRNA-resistant plamids using the TOPO® XL PCR Cloning Kit (Life Technologies) and PCR primers GFP-*EcoRI* (forward) 5'-GATCGAATTCATGGTGAGCAAGGGCGAGGA-3' and NPM1-*NotI* (reverse) 5'-GATCGCGGCCGCTTAAAGCTTCCTCCA-3'. The resultant mutant expression vectors were verified using Sanger sequencing.

2.12.2 Retrovirus production and generation of stable cell lines

To generate GFP-NPM1 siRNA-resistant expressing viruses, 293T cells were seeded on 60 mm^2 culture dishes at a density of 1.2 x 10^6 cells per plate in antibiotic-free 1X DMEM 24hrs prior to transfection. The following day, cells were transfected by calcium phosphate method as described in section 2.3, with transfection reactions that included 2 μg of GFP-NPM1 siRNA-resistant plasmid, 2 μg of pMLVgagpol, 1.5 μg of pHEF-VSV-G and 2.5 μg of pTZ18U. Culture supernatants containing the virus were collected and stored as described in section 2.3 at 48hrs post-transfection. To generate the GFP-NPM1 siRNA-resistant stable cell lines, HeLa cells were seeded on 60 mm^2 culture dishes at a density of 2.8 x 10^5 cells per plate in antibiotic-free media 24hrs prior

to infection. The next day, cells were infected with 1.5 mL of culture supernatants containing the GFP-NPM1 siRNA-resistant expressing viruses with 0.5 mL of antibiotic-free 1X DMEM for 6hrs, washed 3 times in 1X PBS then 2 mL of fresh antibiotic-free 1X DMEM media was added. Selection with 1 μg of puromycin/ml in 1X DMEM was initiated 24hrs after infection. Pooled, stably transduced cells were maintained in 1X DMEM containing 1 μg of puromycin/ml, replaced every 48hrs, at 37˚C, 5% CO_2.

2.13 NPM1 reconstitution experiments

HeLa and HeLa-derived NPM1 siRNA-resistant stable cells were seeded on 6-well plates at a density of 7.25 x 10^4 cells per well in antibiotic-free 1X DMEM 24hrs prior to siRNA transfection. The next day, cells were treated with reagent alone (Mock), siANC or siNPM1 as described in section 2.8. At 24hrs post siRNA treatment, cells were washed 3 times in 1X PBS and either uninfected or infected with VSV-G pseudotyped NL4-3 virus at an MOI of 4 as described in section 2.5. Culture supernatants were collected and assayed for RT activity as described in section 2.6 at 24-and 48hrs post infection. Cells were collected 48hrs post infection, lysed and analyzed by Western blotting as described in section 2.7. To calculate percent virus production, the mean cpm for each infection condition within each experiment was first determined and subtracted from the mean CPM of each corresponding sample lacking virus. The adjusted cpm

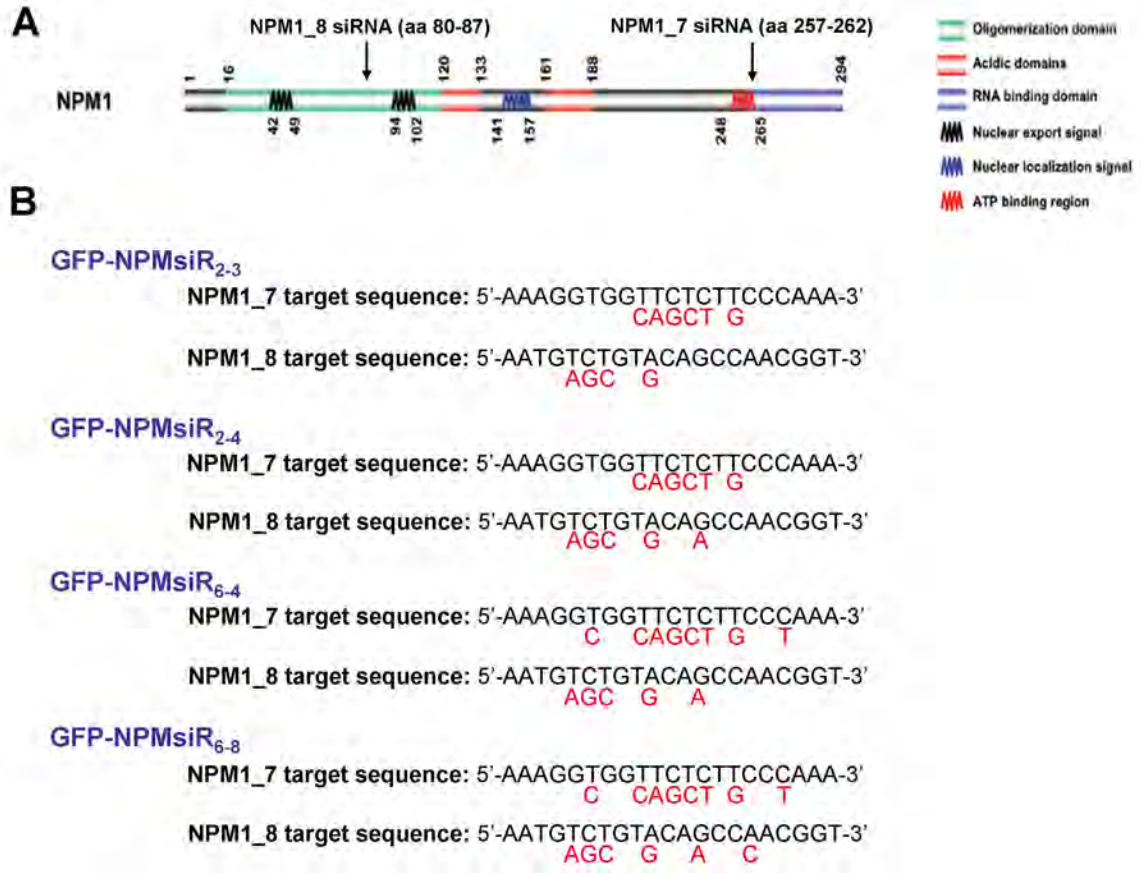


Figure 2-1. Schematic representation of siNPM1 target regions and silent point mutations used to generate GFP-NPM1 siRNA-resistant clones and stable cell lines. (A) Diagram of NPM1 functional domains and the siNPM1 target regions. siNPM1 is an equivalent molar mix of two NPM1-specific siRNA duplexes. NPM1_7 targets nucleotides within amino acids (aa) 257-262 and NPM1_8 targets nucleotides within aa 80-87, indicated by arrows. *Diagram adapted from Mitsuru Okuwaki. J. Biochem (2008).* (B) Depicted are the four GFP-NPM1 siRNA-resistant clones generated (blue) and the silent mutations introduced (red) to confer resistance to the two NPM1-specific siRNA duplex target sequences (black).

mean was normalized to the HeLa Mock condition within the experiment by to obtain the percentage. Data graphs represent the total mean percent virus production from the number of experiments indicated in the figure legends ± SEM. Multiple student T-test was used for statistical analysis (GraphPad Prism6).

2.14 Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

HeLa cells were seeded on 6-well plates at a density of 7.25 x 10^4 cells per well in antibiotic-free 1X DMEM 24hrs prior to siRNA transfection. The next day, cells were treated with reagent alone, siANC or siNPM1 as described in section 2.8. At 24hrs post siRNA treatment, cells were washed 3 times in 1X PBS and either uninfected or infected with VSV-G pseudotyped NL4-3 virus at an MOI of 4 as described in section 2.5. At 24- and 48hrs post infection, culture supernatants were collected and assayed for RT activity as described in section 2.6. In addition, cells were washed with ice cold 1X PBS, trypsinized, and collected into RNase-free 1.5 microfuge tubes and kept on ice. Cells were subsequently counted and 10^4 cells were aliquoted to separate tubes for RT-qPCR analysis. Cells were washed, lysed, RNA isolated, RNA was reverse transcribed (RT) and cDNA amplified using the TaqMan Fast Cells-to-C_T Kit (Life Technologies cat. no. 4399003) according to the manufacturer's recommended protocol, including the added XenoRNA control. Each 20 μL real-time PCR reaction contained 4 μL of cDNA or diluted standard, 10 μL of TaqMan Fast Universal PCR Master Mix, 5 μL of nuclease-free water, and 1 μL of TaqMan Gene Expression Assay primer (Life Technologies) for

the detection of NPM1 (Hs023394979_g1), CLEC2D (Hs00203174_m1) or the DH10β diluted standard control (Ac00010015_a1) expression levels. The TaqMan Cells-to-C_T Control Kit (Life Technologies cat. no. 4386995) included Gene Expression Assay primers to detect the XenoRNA control target and the endogenous control gene β-actin (ACTB) expression levels. Real-time PCR reactions were assayed in duplicate. Amplification and fluorescence measurements were performed using the Applied Biosystems 7500 Fast Real-Time PCR System equipped with SDS v1.4 software under the following cycling conditions: 20 seconds at 95˚C followed by 40 cycles at 95˚C for 3 seconds and 60˚C for 30 seconds. Expression levels of the targeted cDNA were based on a standard curve generated from a DH10β control reference library (Life Technologies, cat. no. 4392545) serially diluted 10-fold for a range of 1 pg/μL to 0.001 pg/μL . Data graphs represent the mean cDNA concentration (pg/μL) from one experiment ± SEM.

CHAPTER III: THE ROLE OF THE NUCLEOLUS IN HIV-1 REV-DEPENDENT VIRAL RNA EXPORT

I conceived and performed all experiments presented in this chapter. Nicholas P. Stone is credited for the Western blots depicted in Figure 3-6C and 3-7C, generated from lysates I provided.

CHAPTER III: THE ROLE OF THE NUCLEOLUS IN HIV-1 REV-DEPENDENT VIRAL RNA EXPORT

3.1 Introduction

The eukaryotic nucleolus is a highly organized non-membrane bound compartment found in the nucleus with the primary function of ribosomal RNA (rRNA) synthesis, processing and ribosomal subunit assembly. The complex process of ribosomal biogenesis occurs within three distinct sub regions of the nucleolus: the fibrillar center (FC), where ribosomal RNA (rRNA) transcription takes place; the surrounding dense fibrillar component (DFC), where pre-rRNAs are processed and modified; and the outer region, the granular component (GC), where rRNAs are further processed and assembled into ribosomes (reviewed in (27, 316)). Research over the past decade coupled with over 700 identified nucleolar associated cellular proteins (6, 7, 200, 282) has demonstrated the nucleolus is more than a ribosome generator; it is a plurifunctional organelle with dynamic protein exchange involved in various aspects of cell biology, including cell stress sensing, cell cycle regulation and signal recognition particle biosynthesis (27, 245, 328).

Since the nucleolus plays a central role in numerous cellular processes, it is then not surprising that the majority of viruses have been found to utilize the nucleolus and/or its associated proteins during viral replication (reviewed in (135, 152, 153, 327)). Some viruses, such as the borna disease virus and the hepatitis delta virus, use the nucleolus as the site for viral RNA (vRNA) transcription and replication (159, 203, 267). Others recruit nucleolar proteins to alternative locations for virus replication. For example, both

poliovirus and hepatitis C virus recruit nucleolin to the cytoplasm to stimulate translation (166, 324, 337), while upstream binding factor (UBF) is relocated to the DNA replication centers of adenovirus and herpes simplex virus 1 (193, 296). Furthermore, many viral proteins localize to the nucleolus either through interactions with nucleolar proteins and/or nucleolar localization signal(s) (NoLS) usually embedded within an overlapping nuclear localization signal (NLS) and RNA binding domain (RBD) (33, 66, 95, 184, 197, 198, 269).

Nucleolar localization of viral proteins has been demonstrated to be functionally coupled to processes such as virus assembly, vRNA processing, genome packaging, ribonucleoprotein formation and vRNA export. The adeno-associated virus (AAV) Cap (capsid) and Rep (replication) proteins localize to the nucleolus, where Cap assembles and interacts with nucleolar protein NPM1 (nucleophosmin 1, B23) through Rep, a step necessary for genome encapsidation and export (22, 171, 331). Additionally, the influenza A NP (nucleoprotein) requires nucleolar localization for vRNA synthesis and ribonucleoprotein complex formation (250). A nucleolar trafficking step is also required for the herpesvirus saimiri nucleocytoplasmic shuttling protein ORF57 where it recruits human TREX (hTREX) proteins to the nucleolus for vRNA export (33). Interestingly, when ORF57 nucleolar localization is abolished but RNA binding maintained, ORF57 is unable to export vRNA to the cytoplasm and this inhibition is rescued only when an alternative NoLS is introduced, thus suggesting vRNA processing and/or ribonucleoprotein export complex formation occurs in the nucleolus (33). Similarly, the Kaposi's sarcoma associated herpesvirus (KSHV) ORF57 homologue also requires

nucleolar trafficking for vRNA export (30, 32). Moreover, there is evidence the KSHV ORF57 ribonucleoprotein complex formed in the nucleolus has additional roles downstream of export, in the enhancement of vRNA translation (31).

Nucleolar localization and trafficking has also been demonstrated to play a role in HIV-1 replication (77, 225, 226, 227, 346). During replication, HIV-1 produces a long primary transcript, which is either further processed by splicing machinery to produce an intronless, fully spliced vRNA or retains some or all introns to produce partially spliced or unspliced transcripts, respectively. All transcripts are exported from the nucleus; however the intron-containing vRNAs are exported through a distinct pathway requiring the viral protein Rev and are therefore termed Rev-dependent vRNA. Several groups have reported HIV-1 vRNA localization within the nucleolus (46, 225, 274). Romanov *et. al.* detected intron-containing subgenomic *gag* mRNA in transiently transfected HeLa cells constitutively expressing Tat by gold antibody labeling and *in situ* hybridization then visualized by electron and light microscopy respectively (274). The nucleolar localization of vRNA was also detected by electron microscopy using *in situ* hybridization and several overlapping DNA probes of *in vitro* infected lymphocytes, a natural target of HIV-1 (46). Perhaps the most compelling experiments, and the first to demonstrate a functional role for vRNA nucleolar localization, were performed by the Rossi group. They reported that lymphocytes stably transduced with a nucleolar localized hammerhead ribozyme designed to site-specifically cleave a conserved region within all HIV-1 RNA transcripts inhibits HIV-1 virus production for sustained periods of more than a few weeks (225). Together these results suggest a previously unidentified

nucleolar localization step for viral RNA during HIV-1 replication. However, these experiments failed to distinguish the class(es) of viral RNAs that localize to the nucleolus or establish how their nucleolar localization is mediated. Moreover, the temporal dependence of nucleolar localization of the vRNAs during replication has yet to be determined.

In addition to viral RNA localization to the nucleolus, it is well established that the essential HIV-1 protein Rev localizes to this compartment (66, 91, 155, 156, 212). Rev functions post-transcriptionally to control the nucleocytoplasmic export of the intron-containing unspliced and partially spliced viral RNA (155, 260). Rev contains two functional domains, an arginine rich motif (ARM) and an effector domain, both of which are necessary to mediate export (155, 156, 212, 260). The ARM domain not only confers sequence-specific binding to a stem-loop structure located within unspliced and partially spliced RNA, termed the Rev Response Element (RRE), it also contains the overlapping NLS/NoLS (66, 155, 156, 212). The effector domain contains a leucine-rich nuclear export signal (NES) that interacts with the cellular proteins CRM1, Ran-GTP and other cofactors to facilitate export of RRE-containing RNAs (13, 21, 99, 106, 155, 201, 202, 275, 336). Several studies have reported that Rev can induce the relocalization important export factors CRM1, Nup98 (nucleoporins) and Nup214 from the nucleus to the nucleolus (70, 161, 178, 271, 346). Furthermore, Rev multimerizes in the nucleolus and can actively bind and translocate a nucleolar targeted RRE when localized in this compartment, demonstrating Rev is functional while in the nucleolus (41, 70). Therefore, it can be hypothesized that the nucleolus is the site for Rev export complex formation.

Taken together with the observation that viral RNAs may require a nucleolar localization step and the detection of intron-containing vRNA in the nucleolus of cells not producing virus extends this hypothesis to suggest that the nucleolus has a specific role in Rev-mediated vRNA export. This model is further supported by the observations that a nucleolar localized Rev binding peptide is able to inhibit HIV-1 replication by sequestering Rev export function (226). However, although some studies have examined intron-containing vRNA localization in the presence or absence of Rev using fluorescent *in situ* hybridization *in vivo*, none used viral infection to monitor Rev influence on localization and none have detected the nucleolus (or regions within) as a localization point (195, 213, 343).

Rev nucleolar localization is mediated by an overlapping NLS/NoLS/RBD (aa positions 34 to 50, 5' TRQARRNRRRRWRER 3') (66, 155, 156). Nucleolar accumulation occurs early after Rev expression (91) and requires continued synthesis of rRNA (75, 91). Moreover, several studies have suggested that this nucleolar localization is dependent upon interaction with cellular NPM1 (100, 204, 229, 230, 294, 306). NPM1 is an abundant, multifunctional nucleolar shuttling phosphoprotein that has nucleic acid binding (DNA and RNA), ribonuclease and molecular chaperone activities carried out by distinct functional regions (151). Interaction of Rev with NPM1 has been mapped to a short sequence required for nucleolar localization in the NLS (aa 37-47) (304) while NPM1 interacts with Rev in its N terminal molecular chaperone region and a putative secondary site (305). However, studies examining the NPM1: Rev interaction has been limited to *in vitro* analysis and *in vivo* reports have failed to demonstrate a direct

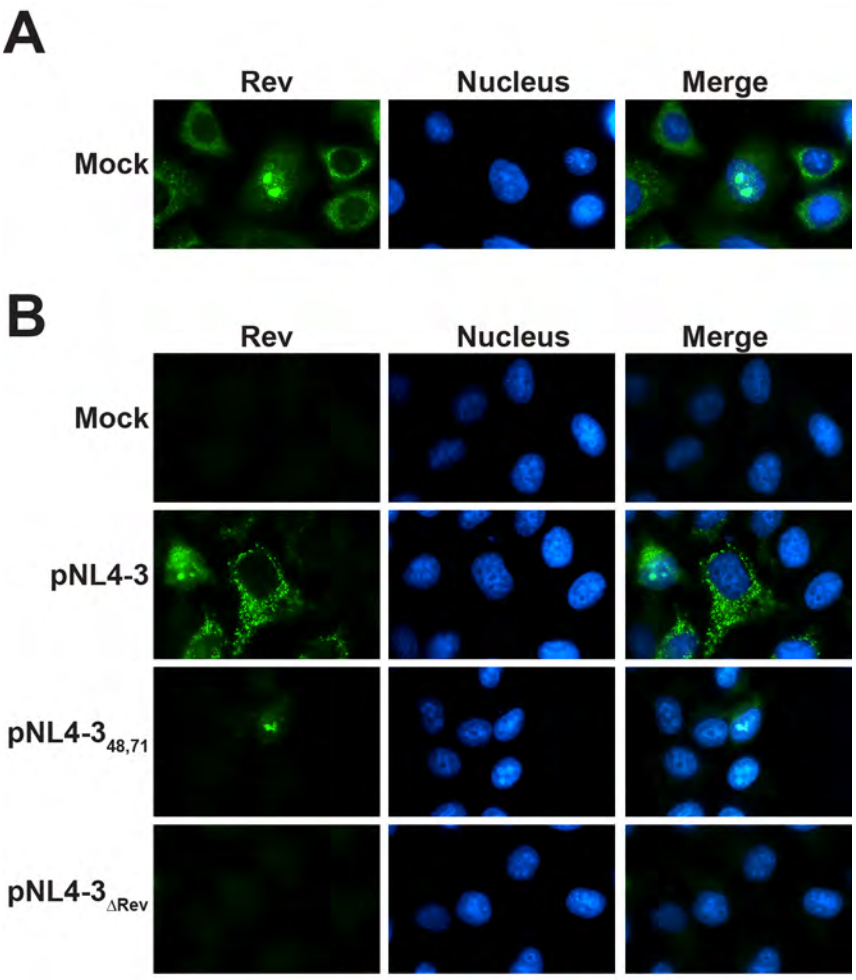
interaction (204, 229, 230, 304, 305, 306). One model for Rev nucleolar localization is that NPM1 mediates the import of Rev from the cytoplasm to the nucleolus (100, 306). However, other cellular factors, including 5 importin β family member proteins and transportin, have also been identified in the role of binding REV NLS for nuclear import (10, 143, 148, 162, 315). It has been suggested the use of transportin is the dominant mode for import (162), while other reports show import factor use is cell type dependent (143). Thus, the role of NPM1: Rev interaction remains to be demonstrated. More specifically, whether NPM1 mediates the nucleolar localization of Rev or has a role in the nucleocytoplasmic shuttling of Rev during Rev-mediated vRNA export has not been examined.

Here, I investigate whether the nucleolus is a site for intron- containing vRNA localization and/or plays a role in Rev-mediated vRNA export. I have analyzed the temporal and intracellular distribution of vRNAs containing site-specific mutations in the RRE post-infection, using coupled fluorescence *in situ* hybridization and indirect immunofluorescence (FISH-IF). This experimental approach was also used to analyze the intracellular distribution of HIV-1 RNA in cells infected with wild type or Rev-deficient virus. In addition, I have examined the role of NPM1 in the nucleolar localization of Rev and Rev-mediated vRNA export *in vivo* using a RNA interference (RNAi)-based approach, a first utilizing this strategy.

3.2 Results

3.2.1 Localization of intron-containing viral RNA is coincident to the nucleolus and does not require Rev expression

Studies have suggested nucleolar localization of HIV-1 vRNA is an essential step during viral replication (225). Although it has been speculated that Rev mediates intron-containing vRNAs to the nucleolus prior to export, the identity of the vRNAs that localize to the nucleolus, how their nucleolar localization is mediated or the temporal dependence of nucleolar localization during replication has yet to be demonstrated. To investigate vRNA localization in the context of virus replication and the dependence of Rev on localization, I utilized a wild type proviral clone (pNL4-3), a proviral clone with two point mutations in the RRE that confers higher affinity Rev binding (pNL4-$3_{48,71}$) and a Rev-deficient provirus (pNL4-$3_{\Delta Rev}$) expressed in HeLa cells (16, 36). To compare Rev localization in the context of viral replication, I used HeLa-LAV cells, a HeLa-derived cell line that is stably infected with the HIV-1_{BRU} (LAV) strain thus producing all viral proteins and releases infectious virus particles as a positive control (17, 18). HeLa-LAV cells were fixed in 100% methanol, immunostained for Rev and analyzed for the intracellular localization by indirect immunofluorescence microscopy. I observed that Rev exhibited two localization phenotypes, either concentrated in the nucleolus or as punctae distributed in the cytoplasm, with a concentration near the perinuclear region, consistent with previous reports (Figure 3-1A) (91, 155). Similar localization of Rev was observed in HeLa cells transfected with either pNL4-3 or pNL4-$3_{48,71,}$ thus confirming proper Rev localization when these proviral clones were expressed (Figure 3-1B, second and third panel respectively). Rev was not detected in the reagent only (Mock) transfected negative control or in cells transfected with the Rev-deficient provirus (Figure 3-1B top and bottom panel, respectively). To further confirm the proviral clones were


A
Rev
Nucleus
Merge
Mock
B
Rev
Nucleus
Merge
Mock
pNL4-3
pNL4-3 48,71
pNL4-3 ΔRev

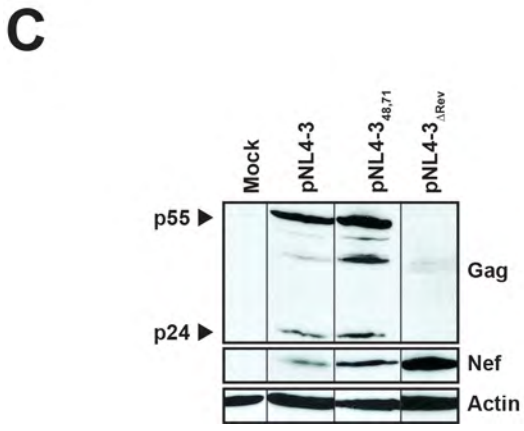

C
Mock
pNL4-3
pNL4-3 48,71
pNL4-3 ΔRev
p55
p24
Gag
Nef
Actin

Figure 3-1. Localization of Rev is both nucleolar and cytoplasmic in cells expressing virus. (A) HeLa-LAV cells were fixed in 100% methanol and immunostained for Rev (green) and the nucleus (blue) using an anti-Rev primary antibody followed by the Alexa Fluor (AF) 564-conjugated secondary antibody and DAPI, respectively. Cells were analyzed by indirect immunofluorescence microscopy. (B, C) HeLa cells were transfected with either reagent alone (Mock), a wild type proviral clone (pNL4-3), a proviral clone with a mutated RRE that confers higher affinity Rev-binding (pNL4-$3_{48,71)}$ or a Rev-deficient provirus (pNL4-$3_{\Delta Rev)}$ and harvested 24hrs post transfection. (B) HeLa cells were fixed in 100% methanol and immunostained as described in A. (C) Cell lysates were analyzed by Western blotting for the detection of the viral proteins Gag and Nef and endogenous Actin as a loading control using anti-Gag, anti-Nef and anti-Actin antibodies, respectively. Data demonstrates two independent experiments.

transcribed and translated, cell lysates were also assayed for viral protein expression and analyzed by Western blot. Intracellular Gag, a Rev-dependent viral protein, and Nef, a Rev-independent viral protein, were detected for both the wild type and RRE-mutated proviral expressing cells (Figure 3-1C). Conversely, barely detectable levels of Gag and increased expression of Nef were observed in cells expressing the Rev-deficient provirus (Figure 3-1C). This observation not only supports expression of pNL4-$3_{\Delta Rev}$, it also suggests increased expression of fully spliced viral transcripts, an expected occurrence in the absence of Rev (104). Collectively, these results demonstrate expression of the proviral and mutant clones, qualifying them for use in further experiments.

Next, I examined the temporal expression of Rev-dependent vRNA to determine the optimal time frame to detect these vRNAs distinctly in subcellular regions of the nucleus. HeLa cells seeded on cover slips were uninfected, infected with VSV-G pseudotyped wild type or Rev-deficient virus. At 6, 12, 24 or 30hrs post infection cells were fixed and analyzed by FISH using a Cy3-conjugated RRE-specific DNA probe and visualized by fluorescence microscopy. I was able to detect intron-containing vRNA as early as 6hrs post wildtype infection as a single, bright nuclear focus in a small percentage of cells (Figure 3-2B top panel). To my knowledge this is the first time RRE-containing vRNA has been visualized this early in infected cells using a FISH technique. Similar bright, single nuclear foci of intron-containing vRNA were observed at 12hrs post infection, consistent with previous observations that also demonstrated these were active sites of vRNA transcription (Figure 3-2B second panel) (195, 343). By 24hrs more than one intron-containing vRNA nuclear foci could be detected in wild type infected

cells and they remained discrete and discernable in localization (Figure 3-2B third panel). Comparable localization was also observed at the same time point in the absence of Rev expression (Figure 3-2C top panel). Conversely, by 30hrs post-infection accumulation of intron-containing vRNA was detected in the cytoplasm and discrete nuclear foci was not observed in wildtype infected cells, demonstrating 30hrs as the saturation point for distinct vRNA foci detection (Figure 3-2B bottom panel). This result is best represented in NL4-3$_{\Delta Rev}$ infected cells at 30hrs, where almost the entire nucleus is filled with intron-containing vRNA (Figure 3-2C bottom panel). It is important to note the absence of foci detection in the Mock infected cells, thus confirming the specificity of the probe (Figure 3-2A). Collectively, these results demonstrate the earliest detection of intron-containing vRNA is 6hrs post infection, while 24hrs is the optimal time point to detect distinct nuclear vRNA foci, with vRNA nuclear saturation occurring 30hrs post infection.

After the time point of 24hrs for optimal discrete nuclear vRNA detection was determined, I examined whether these foci were localized in the nucleolus. To this end, HeLa cells seeded on cover slips were uninfected, infected with a VSV-G pseudotyped wild type or Rev-deficient virus and fixed 24hrs post-infection, then analyzed by FISH followed by indirect immunofluorescence and visualized by fluorescent microscopy. A Cy3-conjugated RRE-specific probe was used to detect the vRNA while an anti-Fibrillarin primary antibody followed by an AF-488 conjugated secondary antibody was used to detect the nucleolus (Figure 3-3A). More specifically, the nucleolar protein Fibrillarin localizes to the fibrillar center (FC) and the dense fibrillar component (DFC) regions of the nucleolus (25, 245). I detected distinct foci of intron-containing vRNA in

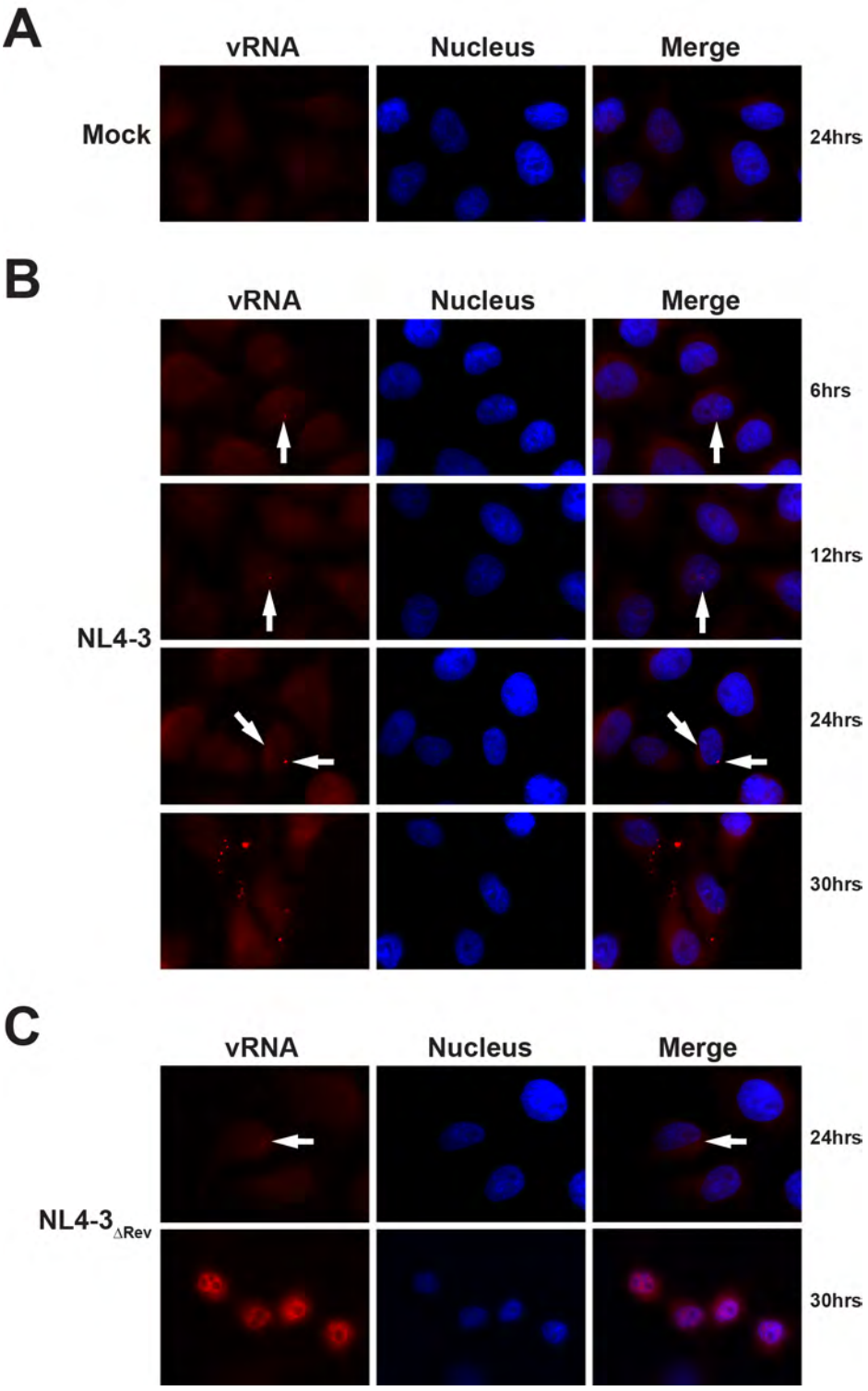

A
vRNA
Nucleus
Merge
Mock
24hrs
B
vRNA
Nucleus
Merge
6hrs
12hrs
NL4-3
24hrs
30hrs
C
vRNA
Nucleus
Merge
24hrs
NL4-3ΔRev
30hrs

Figure 3-2. Localization of Rev-dependent vRNA can be detected as early as 6hrs post-infection as distinct foci in the nucleus. HeLa cells were either uninfected (Mock; A), infected with VSV-G pseudotyped wild type virus (NL4-3; B) or infected with a VSV-G pseudotyped Rev-deficient virus (NL4-$3_{\Delta Rev}$; C) at an MOI of 1 then fixed at the indicated time points in 2% paraformaldehyde/5 mM $MgCl_2$/1X PBS. Rev-dependent vRNA localization (red, foci indicated by arrows) was analyzed by fluorescent *in situ* hybridization using a Cy3-conjugated RRE-specific probe. The nucleus (blue) was detected by DAPI. Arrows indicate discrete vRNA foci detected in the nucleus. Data shown is representative of multiple fields examined in one to two independent experiments.

close proximity to the FC and DFC, however these vRNAs did not co-localize with Fibrillarin, the marker for these regions (Figure 3-3A). Moreover, this localization was not dependent on Rev expression, as a comparable localization of the vRNA was detected for both NL4-3 and NL4-$3_{\Delta Rev}$ (Figure 3-3A, compare middle and bottom panel). Importantly, there were no distinct Cy3-labeled foci detected in the Mock infected negative control cells, further demonstrating specific detection of vRNA (Figure 3-3A top panel). Together, these results indicate that although distinct intron-containing vRNA foci do not localize to the FC or DFC of the nucleolus, they are in close proximity to these regions and this localization is independent of Rev expression.

To extend these results, I used a Cy3-conjugated *gag-pol* oligonucleotide probe to localize full-length vRNA in infected HeLa cells in the presence or absence of Rev expression (Figure 3-3B). Since the Cy3-conjugated RRE-specific probe detects all transcripts containing the *cis*-acting element and my results support previous studies that the observed localization of distinct intron-containing vRNA foci are accumulation of nascent vRNA, then it would be likely that a *gag-pol* intron-specific probe would yield the same localization pattern as the RRE-specific probe (Figure 3-2) (180, 195). Therefore, HeLa cells were seeded on cover slips, then were mock infected or infected with VSV-G pseudotyped NL4-3 or NL4-$3_{\Delta Rev}$ virus, and fixed 24hrs post-infection. Cells were analyzed by FISH using a *gag-pol* RNA-specific probe, followed by indirect immunofluorescence to detect Fibrillarin, and visualized by fluorescent microscopy. I observed the same discrete nuclear foci localized in close proximity to the DFC and FC that also did not co-localize with Fibrillarin in the presence or absence of Rev expression,

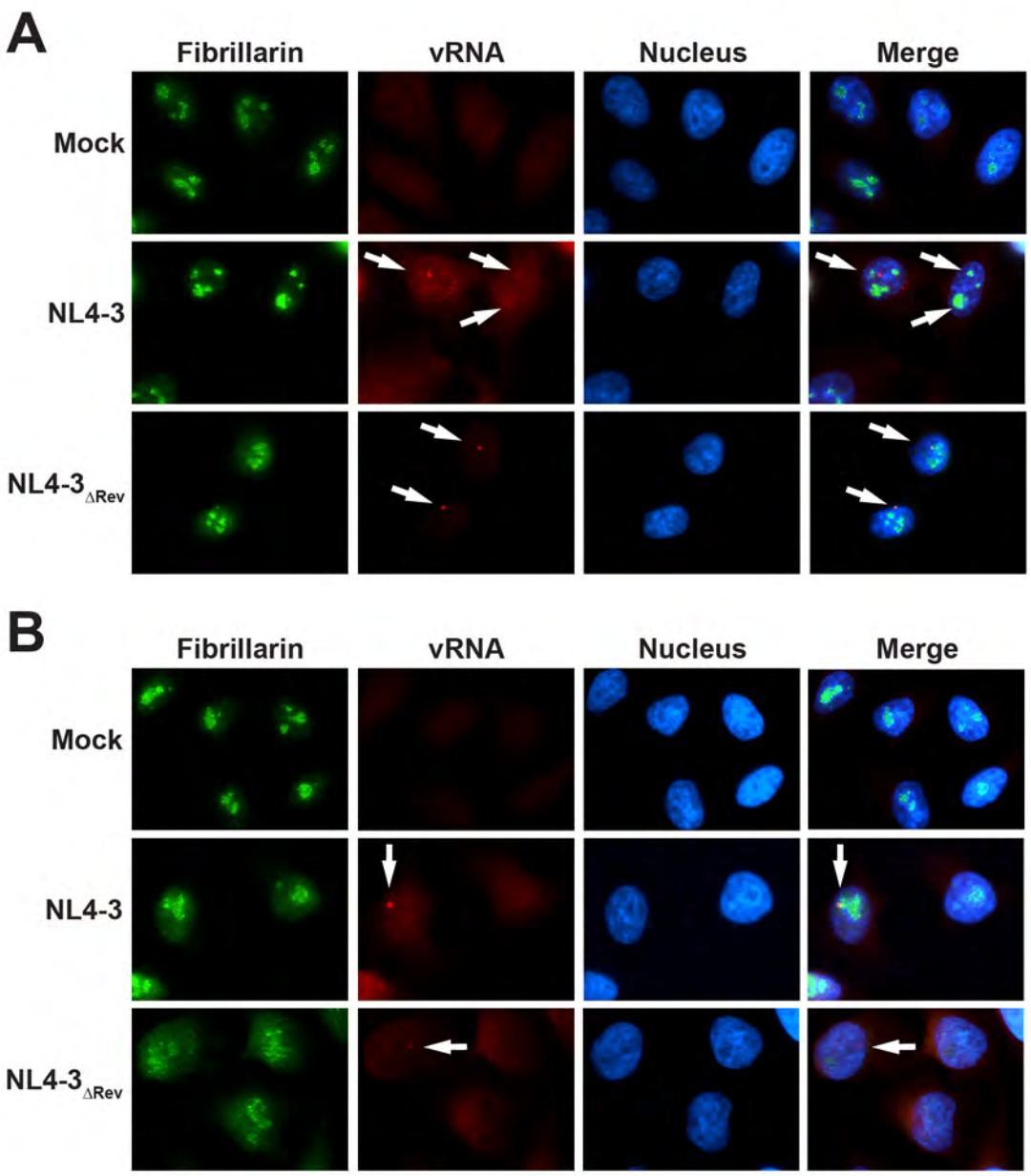


Figure 3-3. Distinct foci of intron-containing vRNA localize in proximity to the FC and DFC of the nucleolus independent of Rev expression and do not co-localize with Fibrillarin. HeLa cells were uninfected (Mock) or infected with a VSV-G pseudotyped wild type (NL4-3) virus or a Rev-deficient virus (NL4-$3_{\Delta Rev}$) at an MOI of

1. At 24hrs post infection, cells were fixed with 2% paraformaldehyde/5 mM $MgCl_2$/1X PBS and analyzed using coupled FISH- IF. A Cy3-conjugated RRE-specific probe (A) or Cy3-conjugated *gag-pol* RNA-specific probe (B) was used to detect vRNA (red, foci indicated by arrows). Immune detection of Fibrillarin (green) was performed using anti-Fibrillarin primary antibody and an AF-488 conjugated secondary antibody. The nucleus (blue) was detected by DAPI. Arrows indicate discrete vRNA foci detected in the nucleus. Data shown is representative of multiple fields examined from one experiment.

which was detected using the RRE-specific probe (Figure 3-3C, compare middle and bottom panels and compare Figure 3-3B and Figure 3-3C). Moreover, no Cy3-labeled foci were observed in the Mock infected negative control (Figure 3-3C, top panel). Therefore, these results further demonstrate the localization of distinct intron-containing vRNA foci is not dependent on Rev expression and are in close proximity to, but do not localize within, the FC or DFC of the nucleolus. In addition, these results support the implication that these discrete foci of vRNA are nascent transcripts.

Although Rev-dependent vRNAs were not detected in the FC or the DFC of the nucleolus, they were localized in proximity to these areas, suggesting they may be located in the GC, the outermost region of the nucleolus. To investigate this possibility, uninfected or infected HeLa cells were fixed 24hrs post infection then analyzed by FISH using the Cy3-conjugated *gag-pol* probe followed by indirect immunofluorescent staining for NPM1, an abundant nucleolar protein found in the GC, and visualized using fluorescent microscopy. Again, I observed no detectable Cy3-labeled foci in the Mock infected negative control cells (Figure 3-4 top panel). Conversely, bright, multiple, discrete foci of Rev-dependent vRNA were localized in close proximity to and adjoining NPM1 in HeLa cells infected with the wild type virus (NL4-3), the virus with a mutated RRE that confers higher affinity Rev binding (NL4-$3_{48,71}$) and the Rev-deficient virus (NL4-$3_{\Delta Rev}$) (Figure 3-4) (16). These results illustrate three exciting insights. First, these results suggest that Rev-dependent vRNA may localize to the GC and/or with NPM1. The second novel insight is that the possible nucleolar localization of the full-length transcripts is not dependent on Rev expression. This is demonstrated two-fold;

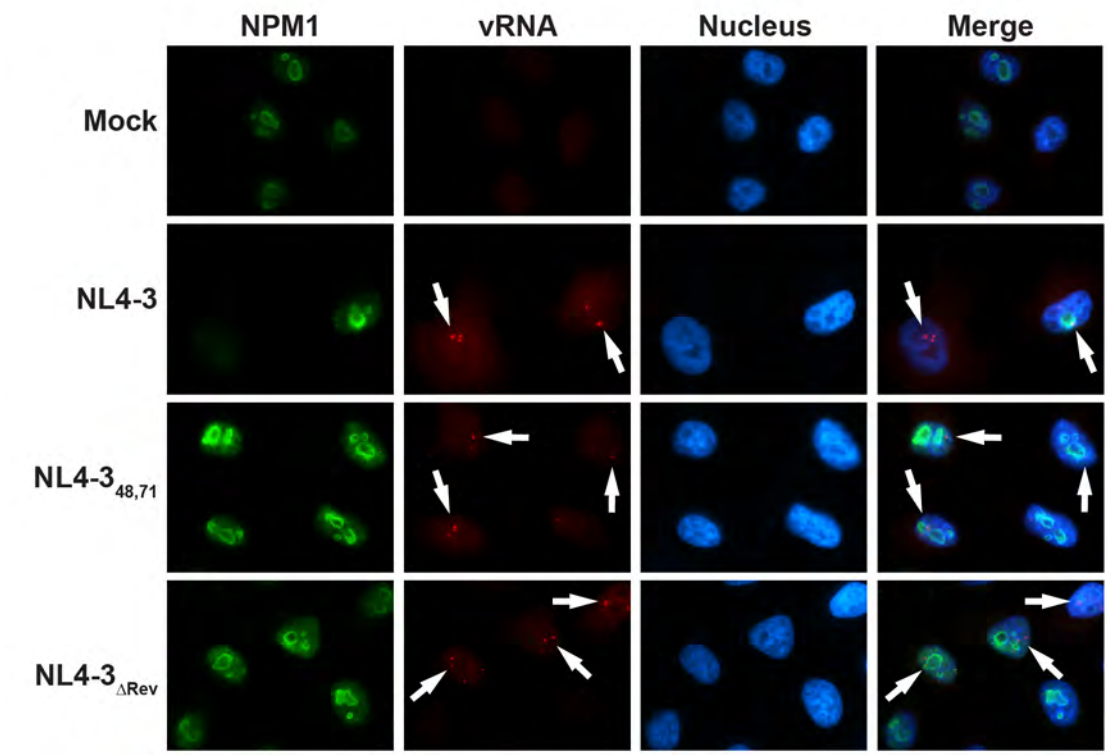


Figure 3-4. Distinct foci of intron-containing vRNA localize coincidently to the GC of the nucleolus and NPM1 independent of Rev expression. HeLa cells were uninfected (Mock), infected with a VSV-G pseudotyped wild type (NL4-3) virus, a virus with a mutated RRE that confers increased affinity for Rev binding (NL4-$3_{48,71}$) or a Rev-deficient virus (NL4-$3_{\Delta Rev}$) at an MOI of 1. At 24hrs post-infection, cells were fixed with 2% paraformaldehyde/5 mM $MgCl_2$/1X PBS and analyzed using coupled FISH- IF using a Cy3-conjugated *gag-pol* RNA-specific probe to detect vRNA (red). Immune detection of NPM1 (green) was performed using anti-NPM1 primary antibody and an AF-488 conjugated secondary antibody. The nucleus (blue) was detected by DAPI. Arrows indicate coincident localization of vRNA foci coincident to the GC. Data shown is representative of multiple fields examined from one experiment.

the localization of the Rev-dependent vRNA foci is similar in NL4-3 and NL4-$3_{\Delta Rev}$ infected cells and the localization of vRNA foci in NL4-$3_{48,71}$ infected cells is similar to both NL4-3 and NL4-$3_{\Delta Rev}$ (Figure 3-4). More specifically, it has been previously demonstrated that the mutations in the RRE that enable increased Rev binding affinity to the RRE results in an increase of Rev export activity, measured by expression of a chloramphenicol acetyltransferase (338) reporter in transiently transfected cells (16). Therefore, one would expect the same mutations in the context of a viral infection would lead to increased export and accumulation of Rev-dependent vRNA in the cytoplasm resulting in increased virus production when compared to wild type virus. I did observe an increase in virus production at 24hrs, measured by standard Reverse Transcriptase (RT) activity assay compared to wild type (data not shown). However, the nuclear localization of the Rev-dependent vRNA was unchanged compared with Rev-dependent vRNA in wild type and the Rev deleted virally infected cells (Figure 3-4). Therefore, these results suggest the coincident localization of intron-containing vRNA to the GC of the nucleolus does not require Rev expression. Lastly, these results further support previous reports that the observed Rev-dependent vRNA foci are nascent transcripts.

3.2.2 Depletion of cellular NPM1 does not effect Rev localization or Rev- mediated vRNA export

It is well established that Rev localizes to the nucleolus and studies have suggested this localization is mediated by the nucleolar shuttling protein NPM1, however, no reports have demonstrated a direct involvement of NPM1 in the localization of Rev *in vivo*. To examine whether NPM1 alone mediates the nucleolar localization of

Rev *in vivo*, I employed an RNAi-based approach. Initially, I determined whether NPM1-specific gene silencing could be achieved in HeLa cells. Based on the results of my RNAi optimization, HeLa cells were either untreated, transfected with reagent only (Mock) or 5 nM of siNPM1 (Figure A1-1). After 48hrs, cells were trypsinized, counted and seeded onto cover slips to be analyzed by indirect immunofluorescence for NPM1 expression. A fraction of the counted cells were lysed and analyzed by Western blot for NPM1 and Actin protein expression. I observed no substantial difference in cell death or cell cycle kinetics in HeLa cells treated with siNPM1 compared with cells untreated or Mock treated (Figure 3-5). Moreover, in untreated and Mock treated cells, NPM1 exhibited similar concentrated localization in the GC of the nucleolus, seen as green ring(s), with detection to a lesser extent in the nucleus, consistent with previous reports (Figure 3-5A, top and middle panels) (245). In contrast, siNPM1 treated cells revealed significant diminution in visible levels of cellular NPM1 in the GC and nearly absent detection in the nucleus (Figure 3-5A, bottom panel). Depletion of NPM1 was confirmed by Western blot analysis. Nearly undetectable levels of NPM1 were observed in the siNPM1 treated cells while similar NPM1 expression was evident in untreated and Mock treated cells (Figure 3-5B). Collectively, these results demonstrate that I am able to achieve effective NPM1 depletion in siNPM1 treated cells.

To investigate whether depletion of NPM1 had an effect on Rev localization, I visualized the subcellular localization of Rev in cells transfected with the previously characterized, functional, enhanced green fluorescent protein fused to the C-terminal of Rev (pRev-GFP) in the absence or presence of NPM1-specifc siRNA (294). Cells were

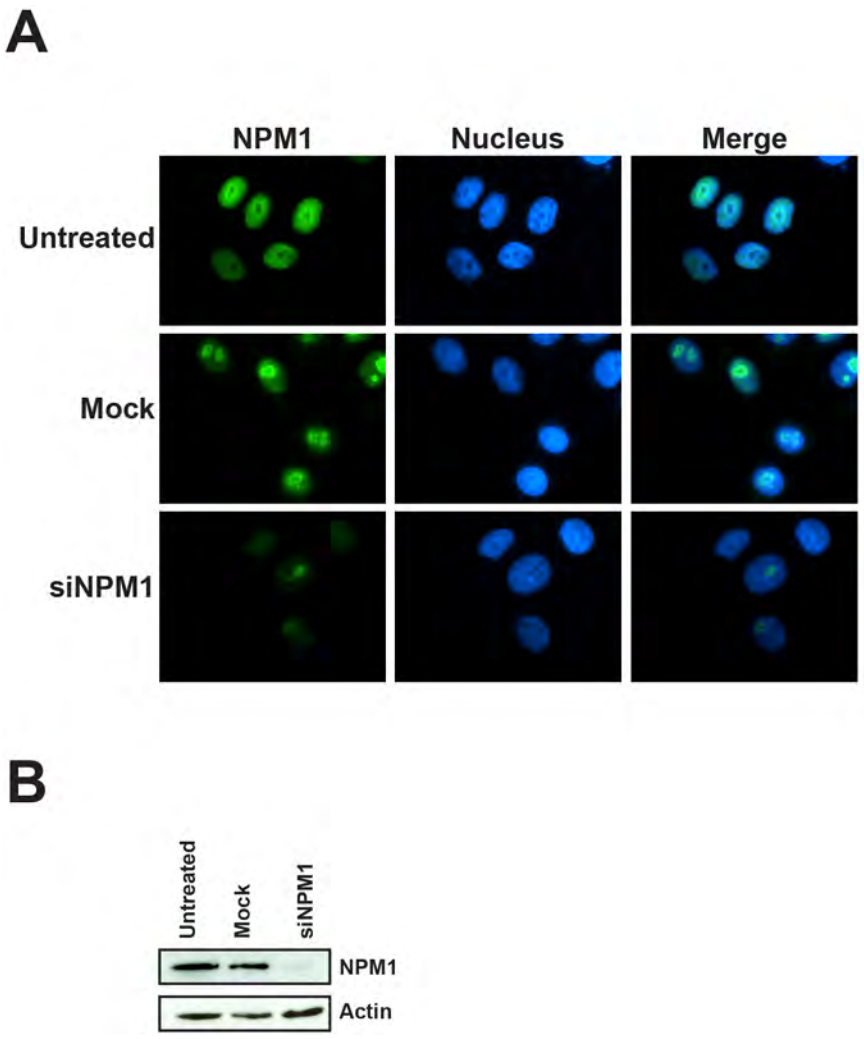


Figure 3-5. Efficient NPM1 depletion is achieved in siNPM1 treated cells. HeLa cells were untreated or transfected with reagent alone (Mock) or 5nM of siNPM1. Cells were harvested 72hrs post-siRNA transfection. (A) Cells were fixed in 4% paraformaldehyde/5 nM $MgCl_2$/1X PBS and immunostained for NPM1 (green) and the nucleus (blue) using an anti-NPM1 primary antibody followed by the AF-488-conjugated secondary antibody and DAPI, respectively. (B) Cell lysates were analyzed by Western blotting for the detection of NPM1 and Actin using anti-NPM1 and anti-Actin antibodies, respectively. Data shown is representative of one experiment performed in triplicate.

Mock treated, treated with a non-silencing control siRNA (siANC) or siNPM1 for 48hrs then seeded onto cover slips to be analyzed by indirect fluorescent microscopy or lysed to be analyzed by Western blotting for the expression of NPM1, Rev-GFP and Actin. Cells were subsequently transfected with a control vector (pBC12/CMV/IL-2, Mock) or Rev-GFP and harvested 24- or 48-hrs later. Nearly undetectable levels of NPM1 were observed in HeLa cells treated with siNPM1, whereas similar basal levels of NPM1 were observed in the Mock and siANC-treated cells after 24-and 48hrs (Figure 3-6 and Figure 3-7). Transfection of Mock or siANC and an expression vector did not effect NPM1 intracellular localization or expression at any time point (Figure 3-6 and Figure 3-7). In these treated cells, cellular NPM1 was observed predominately concentrated in the GC of the nucleolus with some nuclear localization, consistent with my previous observations (Figure 3-6A, B and Figure 3-7A, B). Moreover, Rev-GFP was primarily visualized in the nucleolus and co-localized with NPM1 (Figure 3-6B top, middle panel and Figure 3-7B top, middle panel). However, despite almost complete reduction in intracellular NPM1 by siRNA treatment, demonstrated by Western blotting and indirect fluorescence microscopy, Rev-GFP retained its predominant nucleolar localization 24hrs and 48hrs after transfection (Figure3-6B and Figure 3-7B). These results suggest that NPM1 is not required for nucleolar localization of Rev.

Although I found that depletion of NPM1 did not affect Rev nucleolar localization, it was unclear whether NPM1 participates in the nucleocytoplasmic shuttling of Rev during Rev-mediated vRNA export. To determine whether NPM1 depletion has an effect on Rev-mediated vRNA export, I used a CAT reporter co-transfection assay to

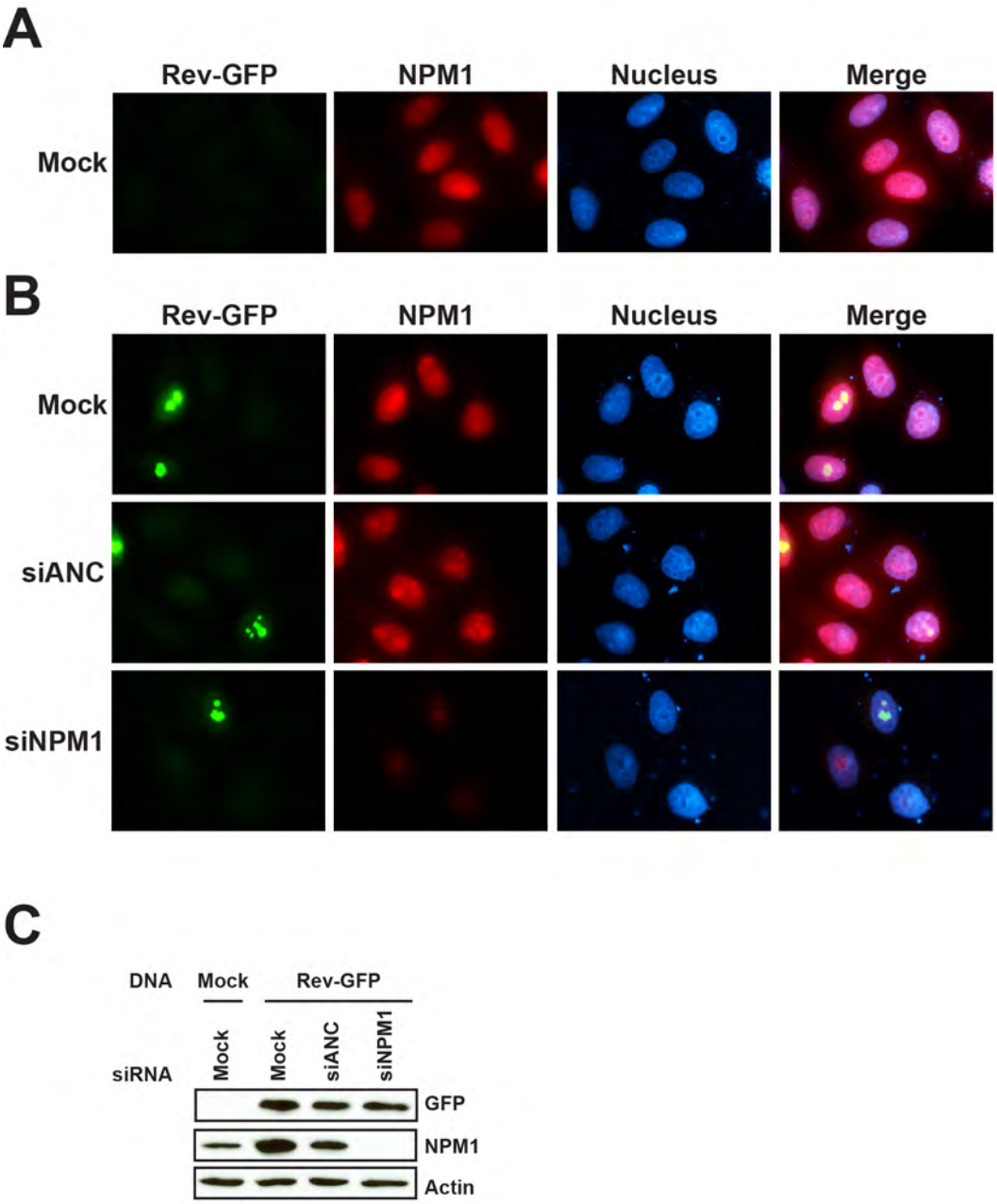


Figure 3-6. Depletion of cellular NPM1 by RNAi does not alter the localization of Rev 24hrs post-transfection. (A, B) HeLa cells were transfected with reagent alone (Mock; A, B top panel), siANC (B middle panel) or siNPM1 (B lower panel). At 48hrs

post siRNA treatment, cells were transfected with a control plasmid (Mock; A) or a Rev-GFP (pRev-GFP, green) expressing plasmid. Cells were fixed with 4% paraformaldehyde/5 nM $MgCl_2$/1X PBS 24hrs after DNA transfection. Cellular NPM1 (red) was analyzed by indirect immunofluorescence with an anti-NPM1 primary antibody followed by an AF-546-conjugated secondary antibody as a reference for Rev-GFP localization and to demonstrate gene-specific knock down. Cell nuclei (blue) were detected with DAPI. Images shown are representative of multiple fields examined in two independent experiments. (C) HeLa cell lysates from A and B were analyzed by Western blotting for Rev-GFP expression and NPM1 gene-specific knock-down using an anti-GFP and anti-NPM1 antibody, respectively. Actin was detected with an anti-Actin antibody as a total protein loading control. Data shown is representative of two independent experiments.

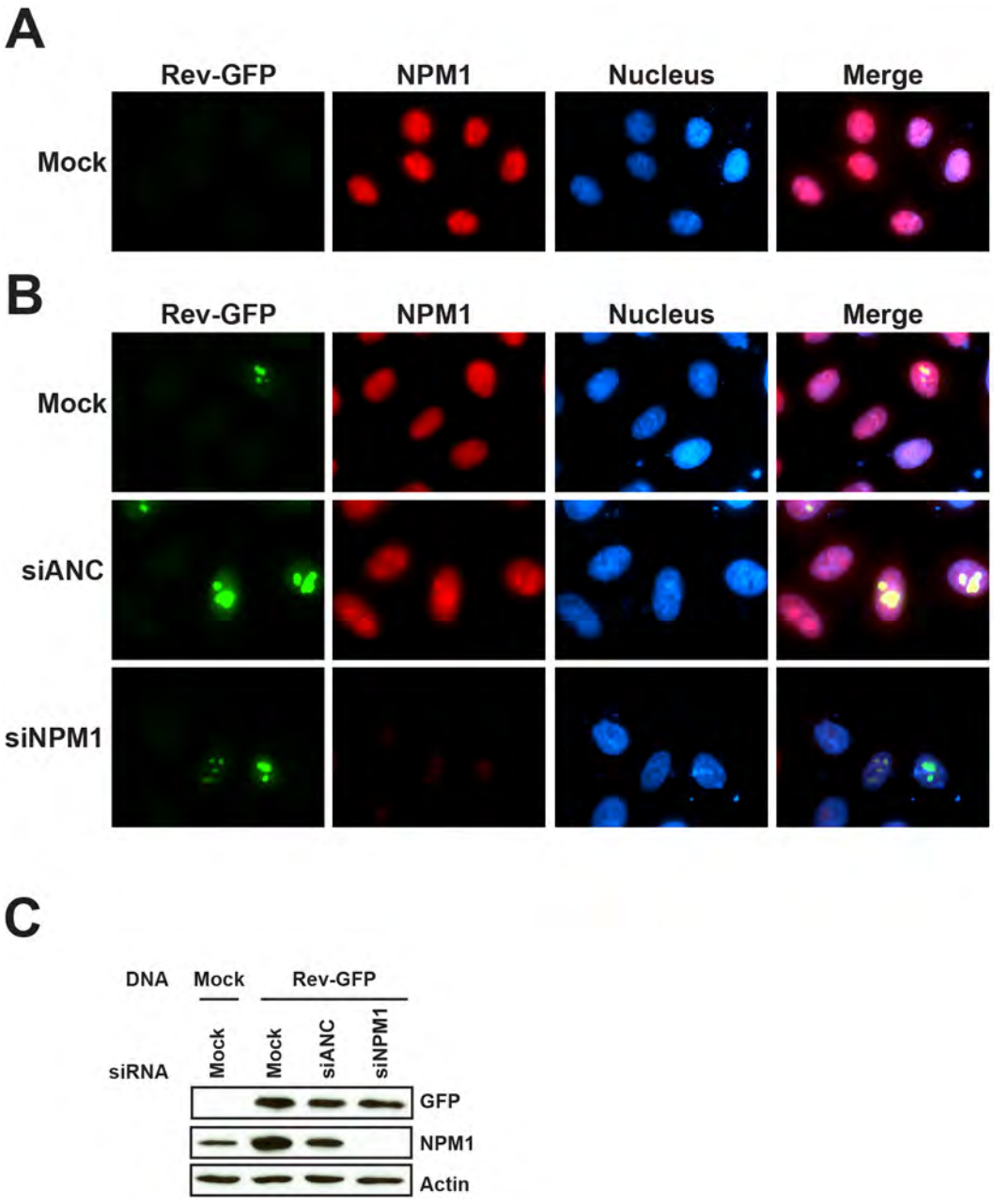


Figure 3-7. Depletion of cellular NPM1 by RNAi does not alter the localization of Rev 48hrs post-transfection. (A, B) HeLa cells were transfected with reagent alone (Mock; A, B top panel), siANC (B middle panel) or siNPM1 (B lower panel). At 48hrs

post siRNA transfection, cells were transfected with a control plasmid (Mock; A) or Rev-GFP (pRev-GFP, green) expressing plasmid. Cells were fixed with 4% paraformaldehyde/5 nM $MgCl_2$/1X PBS 48hrs after DNA transfection. Cellular NPM1 (red) was analyzed by indirect immunofluorescence with an anti-NPM1 primary antibody followed by an AF-546-conjugated secondary antibody as a reference for Rev-GFP localization and to demonstrate gene-specific knock down. Cell nuclei (blue) were detected with DAPI. Images shown are representative of multiple fields examined in two independent experiments. (C) HeLa cell lysates from A and B were analyzed by Western blotting for Rev-GFP expression and NPM1 gene-specific knock-down using an anti-GFP and anti-NPM1 antibody, respectively. Actin was detected with an anti-Actin antibody for a total protein loading control. Data shown is representative of two independent experiments.

monitor Rev activity (156, 157). The CAT reporter plasmid (pCMV128) is derived from the *env* region of HIV-1. Transcripts from pCMV128 do not encode any HIV-1 proteins, but do contain the RRE and the CAT coding sequence within a single intron. When pCMV128 is expressed alone in cells, the intron contained in the transcripts is excised during RNA splicing, which leads to minimal to no measurable CAT enzyme activity in the cytoplasm. However, when pCMV128 is cotransfected with a functional HIV-1 Rev expression vector (pcRev), Rev binds to the RRE present in the transcripts and exports the unspliced RNA to the cytoplasm which allows for the expression of CAT. HeLa cells were either Mock, siANC, or siNPM1 treated for 48hrs then transfected with either a control vector or pCMV128 in the absence or presence of pcRev. Cells were harvested and lysates were prepared 24- or 48hrs later then analyzed for CAT enzyme activity by thin-layer chromatography and protein expression by Western blotting. My Western blot analysis data demonstrate efficient and specific NPM1 depletion was obtained in siNPM1 treated cell lysates at both 24- and 48hrs, irrespective of transfected plasmid expression (Figure 3-8A, C). In addition, I show the expression of NPM1 was comparable for the transfected Mock and siANC treated cell lysates at all time points (Figure 3-8A, C). Evaluation of CAT enzyme activity in cell extracts of Mock and pCMV128 DNA transfected cells also treated with Mock, siANC or siNPM1 revealed no detectable levels of expression at 24- and 48hrs (Figure 3-8A, B). However, CAT enzyme activity was detected in cell lysates co-transfected with pCMV128 and pcRev at all time points. I observed that despite efficient NPM1 depletion in siNPM1 treated pCMV128 and pcRev lysates, there was minimal reduction in acetylation activity compared to pCMV128 and

pcRev Mock and siANC treated lysates at 24hrs and 48hrs (Figure 3-8A, C, compare last lanes with two preceding lanes). To compare the effect of NPM1 depletion on Rev activity, the total percent of acetylation in siRNA treated pCMV128 and pcRev cell lysates was quantified by densitometric analysis of the autoradiographs and found similar mean percent acetylation between cell treatment groups and time points (Figure 3-8B, D). Using a randomized block one-way ANOVA for statistical analysis, there was no significant difference in Rev-mediated vRNA export between treatment groups at 24- or 48hrs ($P= 0.1319$ and $P= 0.1355$, respectively). Therefore, these data suggest NPM1 does not participate in nucleocytoplasmic shutting of Rev during Rev-mediated vRNA export.

3.3 Discussion

In this study, I aimed to elucidate the currently undefined role the nucleolus has in Rev-dependent vRNA export. Specifically, I investigated whether there is a Rev-mediated nucleolar localization step of intron-containing vRNA prior to export. Moreover, I examined whether Rev nucleolar localization and function requires the nucleolar protein NPM1, a previously identified Rev co-factor. I used a coupled fluorescence *in situ* hybridization and indirect immunofluorescence approach to visualize intron-containing vRNA in reference to the nucleolus of infected HeLa cells in the presence or absence of Rev expression. Although several studies have examined Rev-dependent vRNA intracellular localization using this technique, none have selectively labeled the nucleolus for a reference or monitored Rev influence on vRNA localization

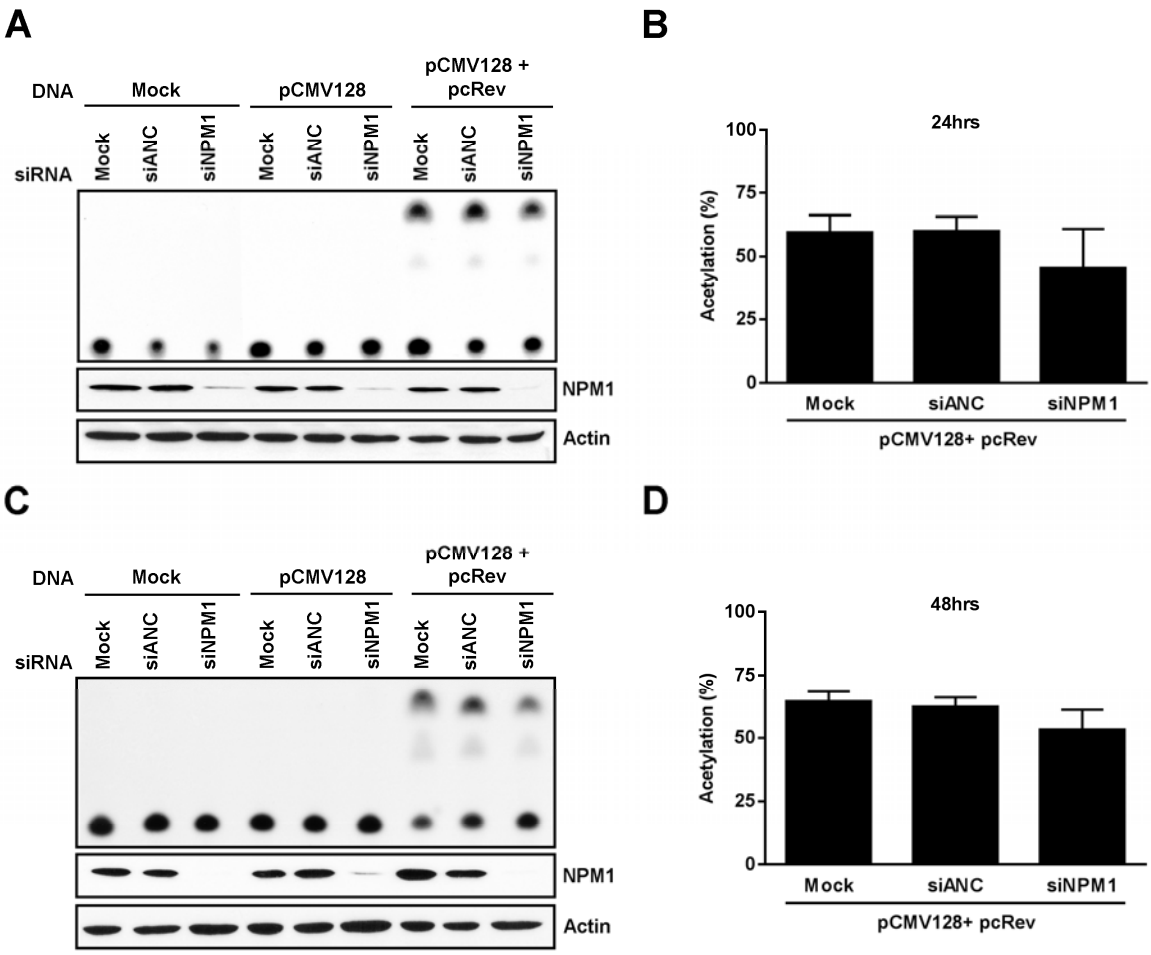


Figure 3-8. Depletion of NPM1 by RNAi does not affect Rev-mediated vRNA export. Functional analysis of Rev was determined by CAT enzyme activity in a transient transfection assay. HeLa cells were transfected with reagent alone (Mock), non-silencing siANC or siNPM1. At 48hrs post initial siRNA transfection, cells were either transfected with a control vector (Mock) or the CAT reporter plasmid pCMV128 in the absence (pCMV128) or presence of pcRev, a HIV-1 Rev expressing plasmid (pCMV128+pcRev). Cell lysates were analyzed for CAT enzyme activity by thin-layer chromatography (A, C; top) and protein expression by Western blotting with anti-NPM1

(A, C; middle) and anti-Actin (A, C; bottom) antibodies at 24hrs (A) and 48hrs (C) after DNA transfection. (B, D) Comparisons of Rev-specific RNA export ability by quantification of chloramphenicol acetylation in the presence of siRNA duplexes. The total percent of acetylation was determined by densitometric analysis of audioradiographs using the Image J software as described in the Material and Methods. Bars represent the mean total percent of acetylation of four (B) and three (D) independent experiments ± standard error of the mean (SEM). A randomized block one-way ANOVA was used for statistical analysis and determined non-significant (NS) P values at 24hrs (P= 0.1319) and 48hrs (P= 0.1355). Data shown in (A) and (C) is representative of four and three independent experiments, respectively.

during viral infection (20, 195, 285, 343). For example, one report utilized a transient cotransfection of Rev and subgenomic plasmid expression vectors (343) while another used a stable cell line expressing *env* mRNA with tetracycline-dependent Rev expression for vRNA localization (285). Consistent with these previous studies, I detected intron-containing vRNA as intense, discrete foci in the nucleus (Figure 3-2B) (20, 195, 285, 343). These distinct intron-containing foci have been previously characterized as an accumulation of nascent viral transcripts (20, 195, 285, 343), with the earliest detection of intron-containing vRNA foci at 12hrs (195, 343). I show for the first time localization of the Rev-dependent vRNA foci as early as 6hrs post infection (Figure 3-2B). My results also demonstrate that the intron-containing vRNA foci remain as discrete, bright foci up to 24hrs, with a saturation point of 30hrs post-infection (Figure 3-2B, also see Figure 3-3 and 3-4). Unlike one previous report, I did not observe a diffuse signal of intron-containing vRNA in addition to the foci in the presence of Rev expression (Figure 3-2A, B) (285) .

Although I did not observe a discernable difference in the localization of the bright, intron-containing vRNA foci in the absence or presence of Rev expression or with a mutated RRE enabling higher Rev binding affinity, I observed coincident nucleolar localization of the intron-containing vRNA foci in these infected cells (Figure 3-3 and Figure 3-4). It is tempting to assign the localization of Rev-dependent vRNAs as a co-localization to the GC of the nucleolus and/or with NPM1 (Figure 3-4). However, since the localization of vRNA and cellular proteins are visualized using a Zeiss Axioplan 2 wide-field fluorescence microscope and the images are taken in a single optical axis (Z)

and specimen plane (X and Y), the images lack sufficient serial spatial depth resolution to definitively conclude this nucleolar localization. Electron microscopy *in situ* hybridization offers the ultrastructural resolution required for specific nucleolar localization. Several studies have examined intron-containing vRNA localization using this method, however the results are inconclusive (46, 64, 274). Two conflicting reports examine intron-containing vRNA localization in the absence or presence of Rev expression in transiently transfected cells, one observed nucleolar localization of intron-containing vRNA in HL-Tat cells, while the other, using COS cells, did not (64, 274). Another study, using infected lymphocytes, detected intron-containing vRNA nucleolar localization, however they did not examine the dependence of Rev expression for Rev-dependent vRNA localization (46). A direct comparison of intron-containing vRNA localization during infection with my wild type, Rev-deficient or enhanced RRE-containing virus using electron microscopy *in situ* hybridization might resolve these observed discrepancies and further strengthen my FISH results.

Of importance, my observation of discrete intron-containing vRNA foci at intranuclear localizations irrespective of Rev expression is consistent with several reports from other groups (20, 195, 285, 343). It has been demonstrated that the intron-containing vRNA foci are an accumulation of nascent transcripts (20, 195, 285, 343). Moreover, these sites were shown to be distinct from Rev-independent vRNA localization (20, 285, 343) and did not localize with the splicing factor SC35 (20, 64, 167, 285, 343). Interestingly, elements within the Rev-dependent vRNA transcript were shown to dictate discrete localization (20, 285, 347). The collective results support a

model in which factors redirect the intron-containing vRNA to distinct regions for alternative processing and expression.

An abundant number of host cellular factors have been identified to influence the processing and transport of Rev-dependent vRNAs, such as Rev-interacting protein (hRIP) (278, 338), several of the DEAD-box RNA helicases (99, 273, 333, 336), peroxisome proliferator-activated receptor-interacting protein with methyltransferase (hPIMT) (335), and nuclear matrix component MATR3 (Matrin 3) (80, 106, 185, 334). Furthermore, some of the known cellular factors bind directly to intron-containing vRNAs and can also localize to the nucleolus. Nucleolar localization has been reported for both U2AF and the heterogeneous nuclear ribonucleoprotein (hnRNP) A1, which are splicing factors that bind to sites found within the nascent vRNAs (85, 192, 240, 310). Also included is the polypyrimidine tract-binding protein associated splicing factor (PSF) (7, 347). PSF is a multifunctional protein involved in several gene expression pathways, including alternative splicing control (109, 286). Recent studies have demonstrated PSF binds to intron-containing vRNA and found to shuttle between paraspeckles, a distinct nuclear compartment that is adjacent to splicing speckles, and the nucleolus (92, 106, 109, 186, 347). A model for the role of PSF in Rev-dependent vRNA processing has been proposed; PSF binds nascent vRNA and redirects the transcripts to an alternative localization for Rev-mediated export (185, 347). I did not examine localization of the aforementioned factors and intron-containing vRNA in the absence or presence of Rev expression. However, my findings of intron-containing vRNA foci localizing to similar discrete nuclear regions support a model involving cellular factors mediating intron-

containing vRNA localization rather than the viral protein Rev (Figure 3-3 and 3-4). Furthermore, the observed localization of the intron-containing vRNAs coincident to the nucleolus, a domain found to be enriched in Rev and Rev binding partners, supports the role of the nucleolus as a site for Rev-mediated export (Figure 3-4). Further vRNA localization and cellular protein analyses are necessary to make such a conclusion. Nonetheless, my localization studies presented here together with data demonstrating Rev is able to multimerize in the nucleolus (77), co-localize with the cellular export factor CRM1 in the this compartment as well as recruit other export co-factors to the nucleolus (78, 346) retain evidence for a nucleolar localization step for intron-containing vRNA during Rev-mediated vRNA export.

As an alternative approach to elucidate the role of the nucleolus in Rev-dependent vRNA export, I examined whether Rev nucleolar localization and function requires the nucleolar protein NPM1. Studies have demonstrated Rev co-localizes and interacts with nucleolar shuttling protein NPM1, and suggested to be the receptor mediating Rev nuclear import and/or nucleolar localization (91, 100, 151, 229, 230, 304, 306). More precisely, research has shown NPM1 binds Rev (100, 304, 305, 306), stimulates the nuclear import of Rev *in vitro* (306) and may be a component of the Rev export complex (204). However, evidence is lacking to demonstrate NPM1 is responsible for Rev import and/or nucleolar localization *in vivo* or established a functional link between a NPM1:Rev interaction, nucleolar localization and Rev function. This is the first report using an RNAi-based strategy to examine NPM1 involvement in Rev nucleolar localization and nucleocytoplasmic shuttling during Rev-mediated vRNA export *in vivo*.

Here, specific siRNA-mediated knock down of cellular NPM1 was achieved, as assessed by indirect immunofluorescence microscopy and Western blotting, using a NPM1 specific antibody (Figure 3-5 through 3-8). Several studies have demonstrated a role for NPM1 in various aspects of cell cycle regulation, suggesting inhibition of NPM1 by RNAi would cause defects in cell cycle progression and cell death (34, 170, 241, 268). However, I observed no discernable difference in cell viability in HeLa cells treated with siNPM1 (Figure 3-5 through 3-8). Consistent with previous reports, I also observed Rev and NPM1 co-localize in the nucleolus (91, 204, 229, 230). Although I was able to achieve efficient and specific depletion of NPM1 expression in siNPM1 treated cells without evident cytotoxicity, I did not observe a localization change of Rev from the nucleolus in NPM1 depleted cells (Figure 3-6 and 3-7). These results strongly suggest that NPM1 does not have a role in the nucleolar localization of Rev. Other studies have demonstrated NPM1 interacts with Rev, however, none examined whether a disruption of this interaction affects Rev localization (151, 229, 230, 304, 306). Therefore, my results are the first report of an examination of NPM1 and Rev localization. Rev has been demonstrated to interact with other nucleolar proteins and requires active rRNA synthesis for nucleolar localization. Given that Rev retains nucleolar localization in NPM1 depleted cells; my results support the premise that other cellular factors or Rev itself mediate this localization. Interestingly, a study showed a redistribution of Rev from the nucleolus to the nucleoplasm and cytoplasm by Nullbasic, a mutant HIV-1 Tat protein, which also redistributed NPM1 in a Rev-dependent manner (204). These data suggest disruption of a specific, common factor for nucleolar accumulation.

I am also the first to investigate whether NPM1 participates in the nucleocytoplasmic shuttling of Rev during Rev-mediated vRNA export *in vivo*. I used a CAT reporter co-transfection assay to monitor Rev export activity in the presence or depletion of cellular NPM1, there was no significant inhibition of CAT activity, at either time point examined, despite efficient cellular NPM1 depletion (Figure 3-8). Thus, these data indicate NPM1 does not participate in the nucleocytoplasmic shuttling (import and export) of Rev during vRNA export. If NPM1 was required for either of these activities, one would expect to detect a noticeable difference in CAT activity in its absence. This reduction has been demonstrated in similar studies examining the effects of other identified Rev-cofactors on Rev-mediated export (e.g. (143, 165, 231). However, it is important to note that I did not examine whether NPM1 participates in Rev-mediated export in the context of viral replication. Furthermore, my studies presented here do not address whether NPM1 has a role in other identified Rev functions, such as vRNA localization, utilization or translation. Therefore, further investigation is necessary to delineate whether NPM1 has a role in Rev function, and is explored in the following chapter.

CHAPTER IV: THE ROLE OF NPM1 IN HIV-1 VIRAL REPLICATION

I conceived and performed all experiments presented in this chapter. Nicholas P. Stone is credited for generating GFP-NPM1siR$_{6\text{-}4}$ and GFP-NPM1siR$_{6\text{-}8}$ by site directed mutagenesis using the mutagenesis primers I designed. Nicholas P. Stone cloned the GFP-NPMsiR mutants into pCX4pur using my cloning strategy and assisted me in establishing the stable cell lines. Nicholas P. Stone is also credited for the Western blot depicted in Figure 4-5C, generated from lysates I provided.

CHAPTER IV: THE ROLE OF NPM1 IN HIV-1 VIRAL REPLICATION

4.1 Introduction

From transcription initiation in the nucleus to its cytoplasmic end, cellular messenger RNAs (mRNAs) are associated with RNA-binding and other proteins, forming ribonucleoprotein particles (mRNPs) (reviewed in (89, 128, 235, 270)). The composition and arrangement of these proteins on mRNA is dynamic, with protein exchange occurring at all steps of RNA metabolism. As a result, these proteins drive the functional fate of the cognate mRNA through interactions with the cellular machinery. These interactions not only promote pre-mRNA processing, but continue through the transport out of the nucleus to the cytoplasmic stability, translation and/or localization of the mRNAs (reviewed in (89, 128, 235, 270)). Thus, the nuclear history of the mRNA can affect its fate in the cytoplasm.

HIV-1 produces a primary transcript (pre-mRNA) from the integrated proviral genome that encodes all the necessary information to generate every viral component for infectious particle production (111, 116, 266). This capability is accomplished through the utilization of alternative splicing and as a result, gives rise to transcripts that contain none, one or all introns. Thus, HIV-1 must exploit a variety of cellular post-transcriptional gene expression mechanisms to regulate the pre-mRNA and ensure balanced nuclear export and translation of vRNAs. A necessary step in this process is the export of intron-containing vRNAs, which normally would be retained in the nucleus. HIV-1 is able to overcome this cellular requirement for complete splicing and promote

the export of incompletely spliced vRNA through highly specialized interactions between the *cis*-acting Rev Response Element (RRE) found within the intron-containing vRNAs, cellular proteins and the HIV-1 regulatory protein Rev. Rev contains two functional domains, an arginine rich motif (ARM) and an effector domain, both of which are required for the protein-mediated nuclear export of RRE-containing vRNA (155, 156, 212, 260). The ARM domain confers sequence-specific binding to the RRE, while the effector domain contains a leucine-rich nuclear export signal (NES) that interacts with the cellular proteins CRM1, Ran-GTP and other cofactors to facilitate export of the intron-containing RNAs (13, 21, 99, 106, 155, 201, 202, 275, 336).

It is well documented that Rev mediates the nucleocytoplasmic export of the intron-containing vRNAs (73, 155, 213, 260). However, research over the years has demonstrated that nucleocytoplasmic export of vRNAs is not the only function of Rev (reviewed in (138, 141, 174, 299, 301)). Several groups have established a functional role for Rev in the translation of Rev-dependent vRNAs (reviewed in (138, 141)). Specifically, Rev has been shown to enhance polysomal association of different intron-containing vRNAs, leading to their increased protein expression (11, 12, 76, 138, 141, 194, 256). D'Agostino *et al.* demonstrated Gag protein expression was Rev- and RRE-dependent using HL-Tat cells transiently co-transfected with a plasmid expressing unspliced *gag* vRNA in the absence or presence of Rev (76). These results were further extended via fractionation studies of HeLa cells transiently transfected with proviral clones that could or could not express Rev. These experiments revealed a Rev-dependent increase in *gag/pol* and *vpu/env* vRNA association with polysomes, thus demonstrating a

functional link between Rev and translation (76). These results are in agreement with the findings of Arrigo and Chen which showed Rev-dependent expression and enhanced polysomal association of *vpu/env* vRNAs in COS and lymphoid cells (11). Interestingly, these and other studies also illustrated cell type dependent differences in the requirement of Rev for the cytoplasmic accumulation of the intron-containing vRNAs, suggesting Rev failed to interact with specific RNP components in the nucleus (11, 76, 141, 256).

Another functional role that has been identified for Rev is in the enhancement of genomic RNA (gRNA) encapsidation (9, 35, 134, 136, 137). Überla and colleagues observed that the removal of either Rev or the RRE from lentiviral vectors caused no discernible differences in the cytoplasmic accumulation of vRNAs but significantly reduced viral infectivity (208). They further demonstrated in 293T, lymphoid and primary peripheral blood mononuclear cells that the observed reduction in infectivity was due to inefficient packaging of gRNA, thus, linking a Rev: RNA interaction for the selection of full-length RNAs for encapsidation (26, 35, 136). Moreover, this suggests packaging efficiency can be influenced by the cellular pathway used for the export of vRNAs. This hypothesis is further supported by studies of viral replication in murine cells. It has been shown that the subcellular localization of Gag and virus assembly depends on the export pathway of the *gag* vRNA, largely attributed to the inability of one or more factors to function and/or interact properly with the exported vRNP (288, 289, 302). Moreover, the Lever group identified an additional Rev binding site in the leader sequence of Gag, termed loop A (119, 133, 140). They showed two A:G point mutations within the highly conserved loop caused a replication defect, characterized by altered

vRNA trafficking and packaging (134). Interestingly, there were no observed differences between wildtype and mutant virus expression levels of Gag protein (134). Taken together, these results strongly support a model where cytoplasmic vRNA localization and utilization is dictated by post-transcriptional events and vRNP composition.

A number of host cellular factors have been identified to associate with Rev-dependent vRNPs and facilitate post-transcriptional Rev functions. Examples include the Rev-interacting protein (hRIP), which is necessary for the release of the Rev-dependent vRNA from the perinuclear region during Rev-mediated vRNA export (278, 338). Several of the DEAD-box RNA helicases have been demonstrated to have both nuclear export and translation enhancing roles in Rev function (99, 273, 333, 336). Furthermore, some of the known cellular factors bind directly to intron-containing vRNAs and can also localize to the nucleolus. Nucleolar localization has been reported for both U2AF and the heterogeneous nuclear ribonucleoprotein (hnRNP) A1, which are splicing factors that bind to sites found within the nascent vRNAs (85, 192, 240, 310). Moreover, hnRNP A1 has been shown to be relocalized to the cytoplasm during viral infection and form part of the vRNP, stimulating cap dependent translation (232).

The nucleolar protein NPM1 has also been identified to interact with Rev and shown to be a Rev co-factor (100, 304, 305, 306). NPM1 is an abundant nucleolar-cytoplasmic shuttling phosphoprotein that has nucleic acid binding properties (DNA and RNA) and ribonuclease and molecular chaperone activities located in distinct functional regions (151). However, its precise role in Rev function during viral replication has yet to be identified. One study has suggested NPM1 acts as a molecular chaperone for Rev;

however this function has not been demonstrated *in vivo* or in the presence of vRNA (305). Other studies have suggested NPM1 is responsible for the nuclear import and nucleolar localization of Rev (100, 204, 229, 230, 294, 306). Yet, these studies have been limited to *in vitro* analyses and *in vivo* reports did not demonstrate a direct interaction (204, 229, 230, 304, 306). Importantly, these reports were unable to establish a functional link between NPM1 and Rev.

In Chapter 3, I investigated the role of NPM1 in the nucleolar localization of Rev and Rev-mediated vRNA export which, to date, is the only study to examine NPM1 involvement in Rev export *in vivo*. My results indicate that NPM1 did not have a role in Rev-mediated vRNA export. However, this study was limited to a transient transfection reporter-based system to examine Rev function. Notably, it is currently unknown whether NPM1 has a role in Rev-mediated export in the context of viral replication. Furthermore, whether NPM1 has a role in other identified Rev functions, such as vRNA localization, utilization, translation or affects virus production post-transcriptionally has not been explored.

Here, I investigate whether NPM1 has a role in Rev function and/or post-transcriptional events during viral replication. I examined the effect of NPM1 depletion on virus production in cell lines and primary human macrophages using an RNAi-based approach. In subsequent experiments, I established stable cell lines that express siRNA-resistant GFP-NPM1 proteins for use in functional rescue experiments. In addition, I have examined the role of NPM1 in Rev-mediated vRNA localization by analyzing the intracellular distribution of intron-containing vRNAs post-infection, using coupled RNA

fluorescence *in situ* hybridization and indirect immunofluorescence.

4.2 Results

4.2.1 Depletion of cellular NPM1 significantly inhibits virus production in infected cell lines and primary human macrophages

Despite being identified as a Rev interacting partner, a role for NPM1 in Rev function has not been established (100, 304, 305, 306). Surprisingly, to date, no study has examined whether NPM1 has a post-transcriptional role during HIV-1 replication. I employed an RNAi-based approach to investigate a possible role for NPM1 in Rev function and/or other post-transcriptional events in the context of viral replication. I have previously demonstrated the ability to achieve specific, efficient NPM1 gene silencing in HeLa cells (Figure A1-1, Figure 3-5 to 3-9). To determine whether depletion of NPM1 protein expression could affect virus production, HeLa cells were transfected with reagent alone (Mock), 5 nM of a non-silencing control siRNA (siANC) or 5 nM of NPM1-specifc siRNA (siNPM1). After 48hrs of siRNA treatment, cells were infected with VSV-G pseudotyped wild-type virus (NL4-3; MOI= 4). Culture supernatants were collected 24- and 48hrs post infection and quantified for virus production by viral RT enzyme activity assays. Cell lysates were analyzed for NPM1 and HIV-1 Gag protein expression by Western blotting 48hrs post infection. Intriguingly, I observed a substantial reduction in virus production in cells depleted of NPM1 expression 24- and 48hrs after infection (Figure 4-1A). My results revealed a mean of 29% virus production 24hrs post infection and 50% 48hrs post infection (SEM ± 1.3 and ± 4.4, respectively).

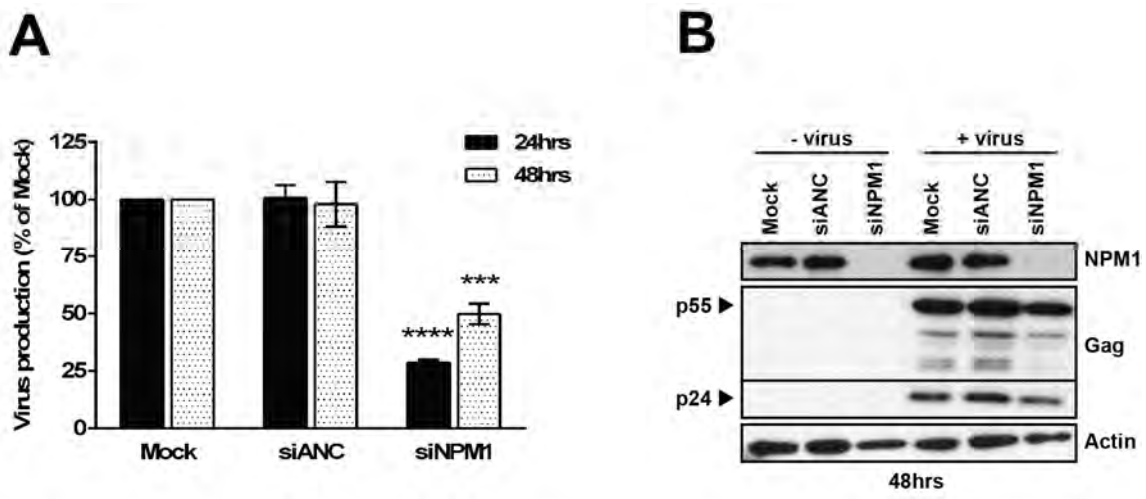


Figure 4-1. Depletion of NPM1 expression by RNAi inhibits HIV-1 production in HeLa cells. HeLa cells were transfected with reagent alone (Mock), 5 nM of siANC or 5 nM of siNPM1. At 48hrs post siRNA transfection, cells were uninfected or infected with VSV-G pseudotyped NL3-4 virus at an MOI of 4. (A) Virus production was determined by quantifying viral RT enzyme activity of culture supernatants 24- and 48hrs post infection as described in Material and Methods. Bars represent the total mean percent of virus production from five independent experiments, four performed in duplicate ± SEM. Multiple student T-test analysis determined significant P values at 24hrs (****, P≤ 0.0001) and 48hrs (***, P≤ 0.001). (B) Cell lysates were analyzed for NPM1 gene-specific knock-down and intracellular Gag expression by Western blotting 48hrs post infection with an anti-NPM1 and anti-Gag antibody, respectively. Actin was detected with an anti-Actin antibody as a total protein loading control. Data shown is representative of cell lysates prepared from experiments in A.

Moreover, multiple student T-test analysis comparing virus production in siNPM1 treated cells to siANC treated cells, determined this reduction to be significant, with P values of $P \leq 0.0001$ at 24hrs and $P \leq 0.001$ at 48hrs. Depletion of NPM1 expression was confirmed by Western blot analysis. Nearly undetectable levels of NPM1 were observed in the siNPM1 treated cells in the absence or presence of virus, while similar NPM1 expression was evident in siANC and Mock treated cells (Figure 4-1B). Interestingly, the intracellular Gag expression levels were only slightly reduced, with no significant qualitative differences in the presence of siNPM1 (Figure 4-1B; compare lane 6 with lanes 4 and 5). The findings of an observed reduction in virus production and nearly unchanged intracellular Gag expression in siNPM1 infected HeLa cells are in agreement with initial experimental results performed in siRNA treated cells transiently transfected with wildtype provirus (Figure A1-2). Furthermore, these results were also recapitulated in siRNA treated HeLa cells infected at a lower MOI (MOI=1; Figure A1-3).

To extend these findings, I examined whether NPM1 depletion could affect virus production in a relevant cell type for HIV-1 pathogenesis, monocyte derived primary human macrophages (MDM). Based on my RNAi optimization data, differentiated MDM were Mock transfected or transfected with 10 nM of siANC or 10 nM siNPM1 (Figure A1-4). After 48hrs of siRNA treatment, cells were infected with VSV-G pseudotyped virus (NL4-$3_{\Delta env}$; MOI= 4) and culture supernatants were analyzed for virus production 24- and 48hrs post infection by viral RT enzyme activity assays. Consistent with results obtained in HeLa cells, I observed a considerable reduction in virus production in NPM1 depleted cells only (Figure 4-2A). Specifically, virus production in

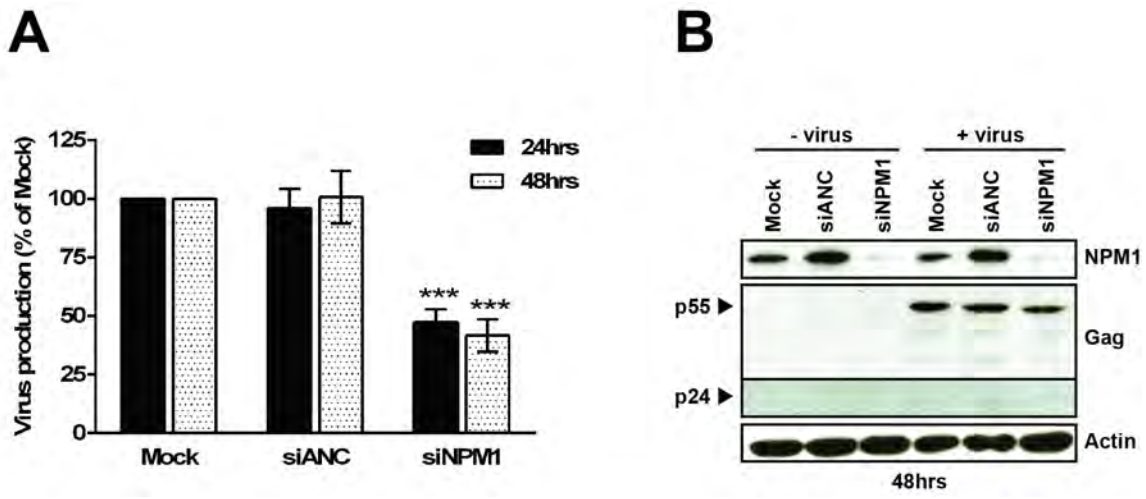


Figure 4-2. Depletion of NPM1 expression by RNAi inhibits HIV-1 production in primary human macrophages. Monocyte-derived primary human macrophages were transfected with reagent alone (Mock), 10 nM of siANC or 10 nM of siNPM1. At 48hrs post siRNA transfection, cells were uninfected or infected with VSV-G pseudotyped NL4-3$_{\Delta env}$ virus at an MOI of 4. (A) Virus production was determined by quantifying RT enzyme activity of culture supernatants 24- and 48hrs post infection as described in Material and Methods. Bars represent the total mean percent of virus production from nine independent experiments ± SEM. Multiple student T-test analysis determined significant P values at 24- and 48hrs (***, $P \leq 0.001$). (B) Cell lysates were analyzed for NPM1 gene-specific knock-down and intracellular Gag expression by Western blotting 48hrs post infection with an anti-NPM1 and anti-Gag antibody, respectively. Data shown is representative of cell lysates prepared from experiments in A.

siNPM1 treated cells was reduced to 47% and 42% at 24- and 48hrs, respectively, a statistically significant reduction when compared with virus production in siANC treated cells ($P \leq 0.001$; 24hrs SEM ± 5.9; 48hrs SEM ± 6.8). Analysis of cell lysates by Western blotting 48hrs post infection confirmed specific and efficient depletion of NPM1 expression in siNPM1 treated cells and, again, revealed only a slight, non significant qualitative reduction in intracellular Gag protein production (Figure 4-2B). Taken together with my data from HeLa cells, these data strongly support a role for NPM1 during virus replication.

Next, I examined whether virus production could be restored in cells depleted of NPM1 expression. To this end, I developed stably transduced cell lines expressing siRNA-resistant GFP-tagged variants of cellular NPM1. The previously characterized wildtype pGFP-NPM1 (97, 98) was used to generate four pGFP-NPM1 siRNA-resistant clones, each containing silent point mutations introduced by site-directed mutagenesis (Figure 2-1). The resultant plasmids were subsequently cloned into the pCX4pur expression vector and sequence verified. Next, VSV-G pseudotyped GFP-NPM1 siRNA-resistant viruses were produced in 293T cells and culture supernatants were collected 48hrs later, then used to infect HeLa cells (Chapter 2). Selection of infected HeLa cells by puromycin was initiated 24hrs later and successful transduction produced four stable cell lines: GFP-NPMsiR$_{2\text{-}3}$, GFP-NPMsiR$_{2\text{-}4}$, GFP-NPMsiR$_{6\text{-}4}$ and GFP-NPMsiR$_{6\text{-}8}$. A more detailed description of how the stable cell lines were generated is provided in Chapter 2. For simplicity, when referring generally to the GFP mutant variants of NPM1, the term GFP-NPMsiR will be used.

Before the GFP-NPMsiR stable cell lines could be used in virus production rescue experiments, characterization of each cell line was necessary. I first examined whether the four stable cell lines exhibited correct localization of their respective GFP-tagged NPM1 proteins. To compare the localization of GFP-NPMsiR expressed in the stable cell lines, I used HeLa cells transiently transfected with wildtype pGFP-NPM1. Transfected HeLa and GFP-NPMsiR stable cell lines were seeded on cover slips and fixed 24hrs later. Cells were stained with DAPI and the intracellular localization of GFP-NPM(196) was analyzed by fluorescence microscopy. I observed bright, concentrated nucleolar localization of GFP-NPM1 (Figure 4-3A). In addition, I observed a minor nucleoplasmic and, to an even lesser extent, cytoplasmic localization of the GFP-tagged wildtype protein (Figure 4-3A). Collectively, these observations were consistent with previous reports (97, 98). Bright, concentrated nucleolar localization of GFP-NPMsiR was also observed for all four stable cell lines (Figure 4-3B). Moreover, I visualized GFP-NPMsiR localization in the nucleoplasm and cytoplasm in all cell lines, albeit to a slightly greater degree than observed in the transiently expressed GFP-NPM1. These observations confirmed proper localization of each GFP-NPMsiR protein (Figure 4-3A, B). To further confirm expression of the respective GFP-NPMsiR for each cell line, cell lysates were assayed for protein expression and analyzed by Western blotting. Expression levels of GFP-NPMsiR and cellular NPM1 was determined using an anti-GFP and anti-NPM1 antibody, respectively. I detected similar expression of GFP-NPMsiR in GFP-NPMsiR$_{2\text{-}3}$, GFP-NPMsiR$_{2\text{-}4}$ and GFP-NPMsiR$_{6\text{-}8}$ stable cells; however, GFP-NPMsiR$_{6\text{-}4}$ expression was considerably greater (Figure 4-3C, top panel). Furthermore, an

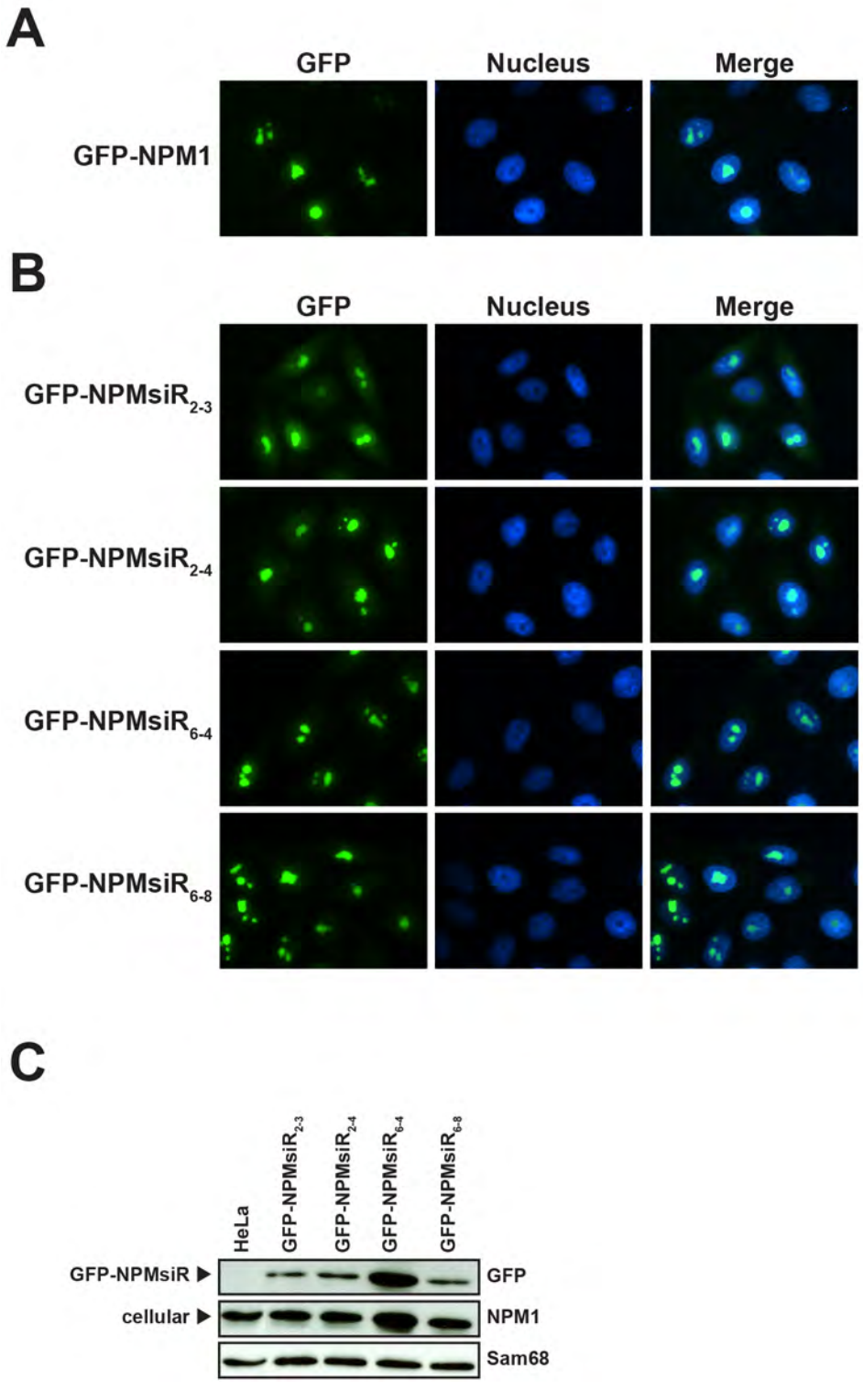

A
GFP
Nucleus
Merge
GFP-NPM1
B
GFP
Nucleus
Merge
GFP-NPMsiR2-3
GFP-NPMsiR2-4
GFP-NPMsiR6-4
GFP-NPMsiR6-8
C
HeLa
GFP-NPMsiR2-3
GFP-NPMsiR2-4
GFP-NPMsiR6-4
GFP-NPMsiR6-8
GFP-NPMsiR ▶
cellular ▶
GFP
NPM1
Sam68

Figure 4-3. GFP-NPMsiR stable cell lines efficiently express nucleolar localized GFP-NPM1 proteins. (A) HeLa cells were transfected with a GFP-NPM1 expressing plasmid (green) and fixed 48hrs later with 4% paraformaldehyde/5 nM $MgCl_2$/1X PBS. Cell nuclei (blue) were detected with DAPI. GFP-NPM1 intracellular localization was analyzed using fluorescence microscopy. (B) GFP-NPMsiR stable cell lines were fixed 24hrs after seeding onto cover slips in 4% paraformaldehyde/5 nM $MgCl_2$/1X PBS. Cell nuclei (blue) were detected with DAPI. GFP-NPMsiR intracellular localization (green) was analyzed using fluorescence microscopy. (C) Cell lysates prepared from experiments shown in B were analyzed for GFP-NPMsiR and cellular NPM1 expression by Western blotting using an anti-GFP and anti-NPM1 antibody, respectively. Sam68 was detected with an anti-Sam68 antibody as a total protein loading control.

enhancement of cellular NPM1 expression was detected in GFP-NPMsiR$_{6-4}$ lysates. This noticeable increase in NPM1 was not due to a difference in total protein loading, as similar expression levels of the control protein, Sam68, was observed (Figure 4-3C, bottom panel). Conversely, GFP-NPMsiR$_{2-3}$, GFP-NPMsiR$_{2-4}$ and GFP-NPMsiR$_{6-8}$ lysates revealed comparable levels of NPM1 expression to that in HeLa lysates (Figure 4-3C, middle panel). Collectively, these results demonstrate comparable stable expression profiles for GFP-NPMsiR$_{2-3}$, GFP-NPMsiR$_{2-4}$ and GFP-NPMsiR$_{6-8}$ cell lines, validating them for use in further experiments. Because the GFP-NPMsiR$_{6-4}$ cell line had notable discrepancies detected for both GFP-NPMsiR and cellular NPM1 expression as well as in virus production capability (Figure A1-5 and discussed below), this cell line was excluded.

Next, I examined whether the GFP-NPMsiR stable cell lines were able to support viral replication. HeLa and GFP-NPMsiR stable cells were uninfected or infected with VSV-G pseudotyped NL3-4 virus (MOI= 4). At 24- and 48hrs post infection, culture supernatants were collected to quantify virus production, while cells were harvested and lysed 48hrs post infection to assay for protein expression. Analysis of culture supernatants for RT enzyme activity showed similar quantities of virus produced from the infected GFP-NPMsiR$_{2-4}$ and GFP-NPMsiR$_{6-8}$ stable cells at 24- and 48hrs (Figure 4-4A). When comparing virus production capability of the stable cells with infected HeLa cells at the same time points, GFP-NPMsiR$_{2-4}$ and GFP-NPMsiR$_{6-8}$ stable cells produced an average of 88% at 24hrs and 119% at 48hrs (average SEM 24hrs ± 28, 48hrs ±19). Thus, these data illustrate virus production in these cell lines parallels what is observed for

HeLa cells. Importantly, infected GFP-NPMsiR$_{2-4}$ and GFP-NPMsiR$_{6-8}$ stable cells also exhibited similar NPM1 and intracellular Gag expression compared with HeLa cells (Figure 4-4B, third, fourth and fifth panel). Analogous expression of GFP-NPMsiR$_{2-4}$ and GFP-NPMsiR$_{6-8}$ was also observed for these cell lines, however, the level of expression detected differed between the two antibodies used to identify the same protein (Figure 4-4B, first and second panel). Specifically, use of the anti-GFP antibody to detect GFP-NPMsiR revealed a greater amount of protein detected than the anti-NPM1 antibody. This suggests the possibility that the three-dimensional folded structures of the GFP-tagged NPM1 could be altered from that of the native form. This insight is further supported by the inability of the mouse monoclonal anti-NPM1 antibody to detect GFP-NPMsiR$_{2-3}$, despite only differing in sequence from GFP-NPMsiR$_{2-4}$ by one nucleotide point mutation (Figure A1-5B, first and second panel; Figure 2-1). The failure to detect expression of GFP-NPMsiR$_{2-3}$ with an anti-NPM1 antibody was one reason this cell line was excluded from additional experiments. Although reports using a GFP-NPM1 protein to examine NPM1 function have not reported experimental difficulties (196, 244), these observations were kept in consideration during additional studies.

The final characterization of the GFP-NPMsiR$_{2-4}$ and GFP-NPMsiR$_{6-8}$ cell lines determined whether the silent point mutations in the expressed GFP-NPMsiR$_{2-4}$ and GFP-NPMsiR$_{6-8}$ proteins rendered resistance to siNPM1-mediated gene silencing. HeLa and GFP-NPMsiR cells were transfected with reagent alone (Mock), 5 nM siANC or 5 nM siNPM1. Cells were harvested and lysed 72hrs post treatment, the time point previously identified as the nadir for NPM1 expression during siRNA treatment (Figure

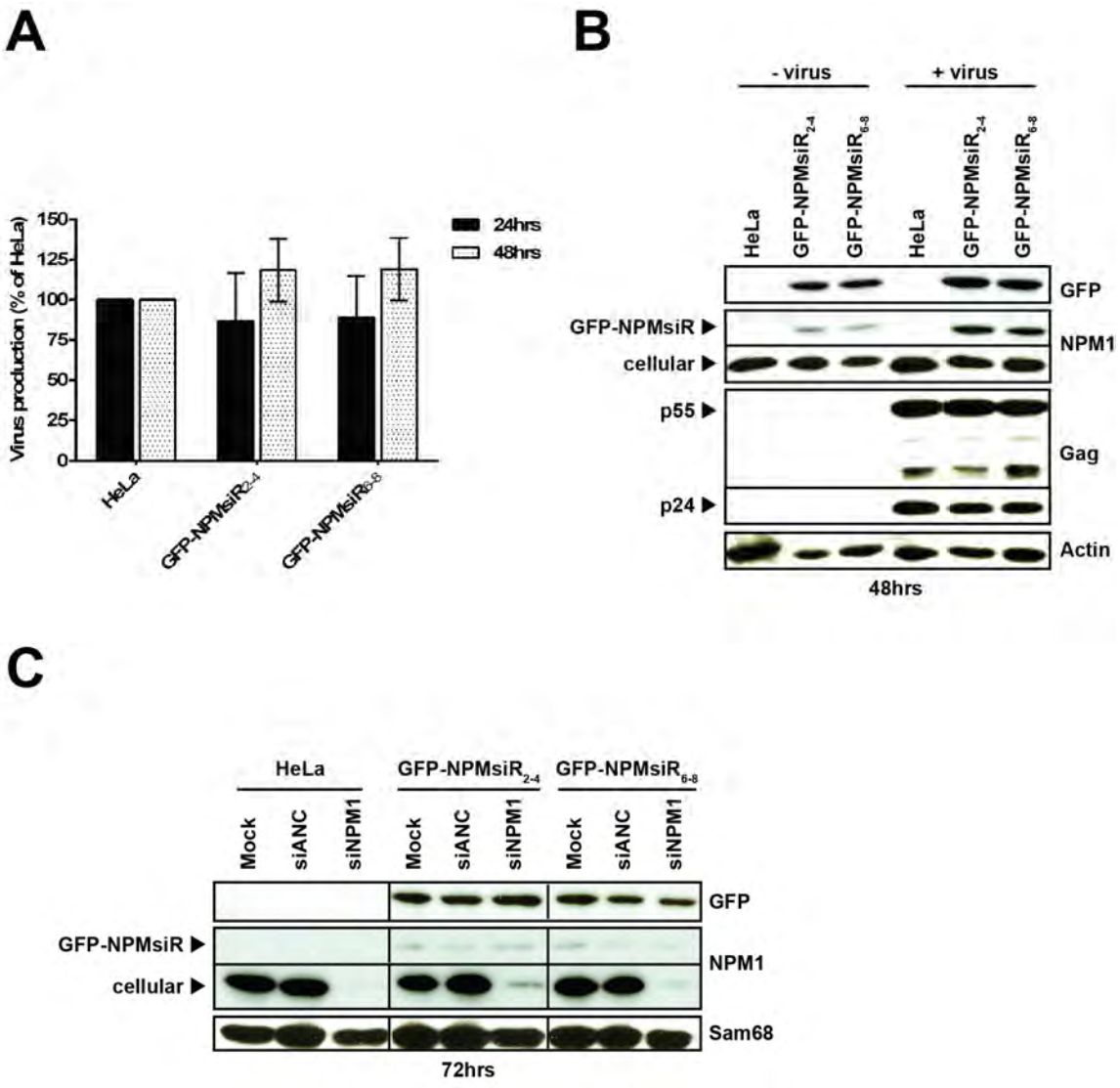


Figure 4-4. GFP-NPMsiR stable cell lines support HIV-1 production and are resistant to NPM1-specific RNAi. (A, B) HeLa and GFP-NPMsiR stable cell lines were uninfected or infected with VSV-G pseudotyped NL4-3 virus at an MOI of 4. (A) Virus production was determined by quantifying viral RT enzyme activity of culture supernatants 24- and 48hrs post infection as described in Material and Methods. Bars represent the total mean percent of virus production from three independent experiments

± SEM. (B) Cell lysates were analyzed for GFP-NPMsiR, NPM1 and intracellular Gag expression by Western blotting 48hrs post infection using anti-GFP, anti-NPM1 and anti-Gag antibody, respectively. Data shown is representative of cellular lysates prepared from experiments in A. (C) HeLa cells or GFP-NPMsiR stable cells lines were transfected with reagent alone (Mock), 5 nM of siANC or 5 nM of siNPM1. At 72hrs post siRNA transfection, cells were harvested, lysed and were analyzed for GFP-NPMsiR and NPM1 expression by Western blotting with an anti-GFP and anti-NPM1 antibody.

A1-1). Consistent with my previous results, I achieved specific, efficient depletion of NPM1 using RNAi. Western blot analysis showed nearly undetectable levels of cellular NPM1 expression for all cell lines, while comparable amounts of NPM1 were detected for Mock and siANC treated cells (Figure 4-4C, third panel). Significantly, I confirmed the expression of GFP-NPMsiR was comparable regardless of siRNA treatment type in the GFP-NPMsi$R_{2\text{-}4}$ and GFP-NPMsi$R_{6\text{-}8}$ cell lines using the anti-GFP antibody (Figure 4-4C, top panel). Of note, I again observed a difference in the level of GFP-NPMsiR expression detected between the anti-GFP and anti-NPM1 antibodies used to identify the protein; there was considerably less GFP-NPMsiR detected with the anti-NPM1 antibody (Figure 4-4C and Figure 4-4B, compare first and second panels). Thus, these results further support the possibility the three-dimensional folded structures of the GFP-tagged NPM1 are altered from the native form. More specifically, the ability of the antibody to recognize the NPM1 antigen may be occluded by misfolding. Nonetheless, the anti-GFP antibody data demonstrate the GFP-NPMsi$R_{2\text{-}4}$ and GFP-NPMsi$R_{6\text{-}8}$ expressing stable cell lines are resistant to siNPM1-mediated depletion, qualifying them for use in the viral production rescue experiments. In contrast, GFP-NPMsi$R_{2\text{-}3}$ cells treated with siNPM1 revealed a decrease in GFP-NPMsiR protein expression, thus providing further rationale for exclusion of this stable cell line from additional experiments (Figure A1-5C, first panel).

Utilizing the characterized and validated siRNA-resistant stable cell lines GFP-NPMsi$R_{2\text{-}4}$ and GFP-NPMsi$R_{6\text{-}8}$, I next investigated whether these cells were able to restore virus production during RNAi-mediated depletion of cellular NPM1. HeLa, GFP-

NPMsiR$_{2-4}$ and GFP-NPMsiR$_{6-8}$ cells were Mock, siANC or siNPM1 treated for 48hrs followed by infection with VSV-G pseudotyped NL4-3 virus (MOI= 4). Culture supernatants were collected 24- and 48hrs post infection to quantify virus production by viral RT enzyme activity assay analysis. Cell lysates were analyzed 48hrs post infection by Western blotting to examine GFP-NPMsiR, NPM1 and intracellular Gag expression. RT enzyme activity assay data revealed analogous percentage of virus production in GFP-NPMsiR$_{2-4}$, GFP-NPMsiR$_{6-8}$ and HeLa cells in the presence of Mock or siANC treatment at 24- and 48hrs post infection (Figure 4-5A and B). However, in the presence of siNPM1 treatment, expression of GFP-NPMsiR$_{2-4}$ and GFP-NPMsiR$_{6-8}$ did not augment virus production. More specifically, despite expression of siRNA-resistant GFP-NPM1, the stable cell lines remained susceptible to a siNPM1-mediated virus replication deficiency. The mean percent of virus production in GFP-NPMsiR$_{2-4}$ and GFP-NPMsiR$_{6-8}$ cells was nearly identical to treated HeLa cells at 24hrs, while at 48hrs the stable cells exhibited marginally less virus production than HeLa cells (Figure 4-5A and B). The inability to rescue virus production in the siNPM1 treated stable cell lines was not due to inefficient expression of the siRNA-resistant forms of GFP-NPM1. Western blot analysis showed comparable quantities of GFP-NPMsiR protein expression in the presence or absence of siNPM1 using the anti-GFP antibody (Figure 4-5C, top panel). Moreover, these results were validated using the anti-NPM1 antibody. Surprisingly, I was able to detect similar levels of GFP-NPMsiR using the anti-GFP and anti-NPM1 antibody (compare Figure 4-5C and Figure 4-4B,C first and second panels). This observed discrepancy can be partially attributed to the order in which the

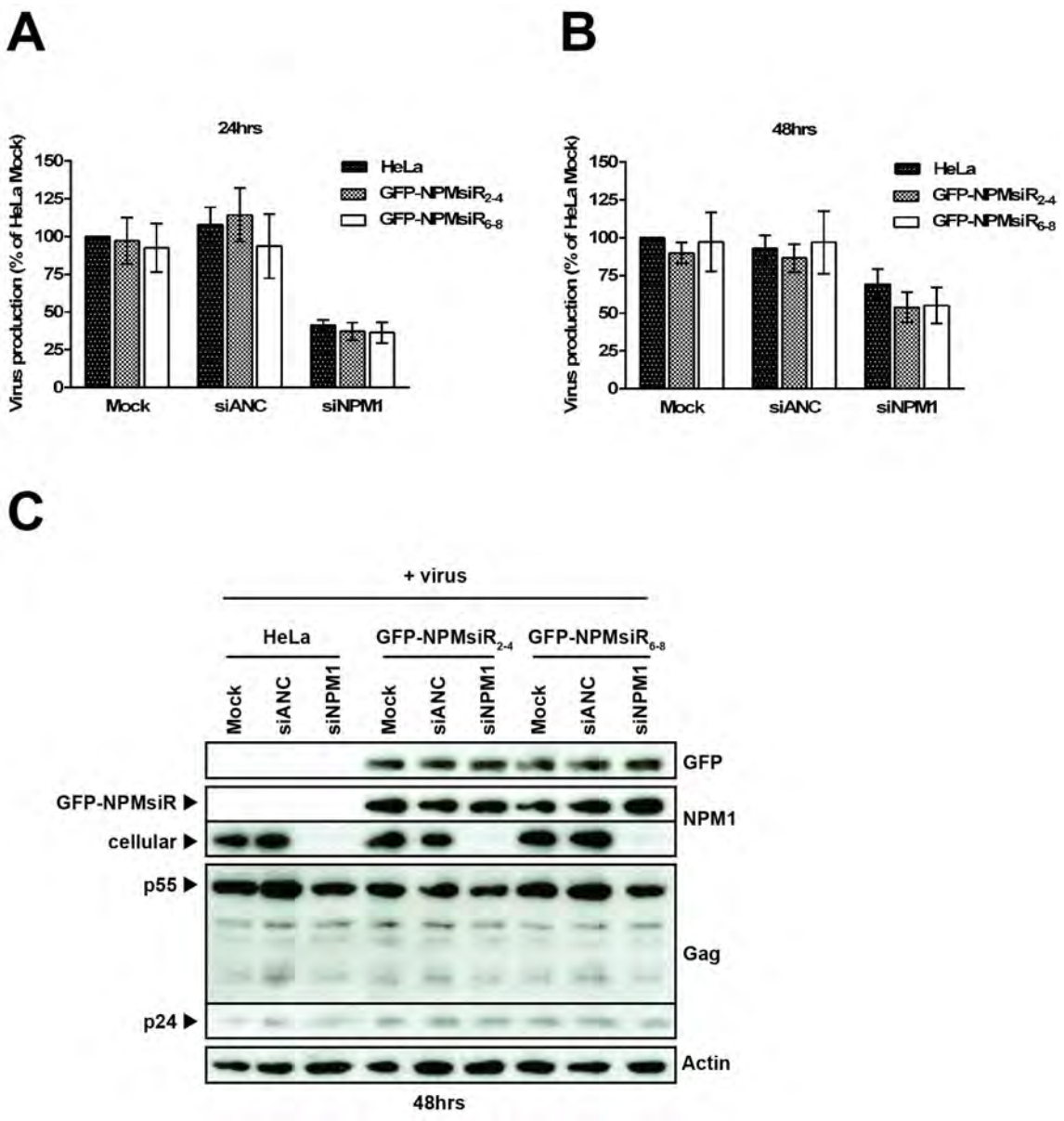


Figure 4-5. GFP-NPMsiR stable cell lines are unable to rescue HIV-1 production in siNPM1 treated cells. HeLa and GFP-NPM1siR stable cell lines were transfected with reagent alone (Mock), 5 nM of siANC or 5 nM of siNPM1. At 48hrs post siRNA transfection, cells were uninfected or infected with VSV-G pseudotyped NL4-3 virus at

an MOI of 4. (A, B) Virus production was determined by quantifying viral RT enzyme activity of culture supernatants 24hrs (A) and 48hrs (B) post infection as described in Material and Methods. Bars represent the total mean percent of virus production from four independent experiments ± SEM. (C) Cell lysates were analyzed for GFP-NPMsiR, NPM1 and intracellular Gag expression by Western blotting 48hrs post infection with anti-GFP, anti-NPM1 and anti-Gag antibodies, respectively. Data shown is representative of cell lysates prepared from experiments in B.

nitrocellulose membranes were immunoblotted with the antibodies. The anti-Gag, anti-GFP and anti-NPM1 are monoclonal antibodies. I observed that when the membranes were immunoblotted with anti-GFP antibody before using the anti-NPM1 antibody, the signal detected for GFP-NPMsiR using the anti-NPM1 was greater. This is most likely due to residual GFP antibody remaining after membrane stripping also being detected by the anti-mouse secondary antibody. Cellular NPM1 was efficiently depleted by siNPM1 in all cell types, while GFP-NPMsiR was observed to have consistent expression in GFP-NPMsiR$_{2\text{-}4}$ and GFP-NPMsiR$_{6\text{-}8}$ cells during siRNA treatment (Figure 4-5C, second panel). Intracellular Gag expression was relatively unaffected by siNPM1 treatment in HeLa, GFP-NPMsiR$_{2\text{-}4}$ and GFP-NPMsiR$_{6\text{-}8}$ cells, consistent with my previous results. Collectively, these data indicate expression of the two GFP-NPM siRNA-resistant proteins, GFP-NPMsiR$_{2\text{-}4}$ and GFP-NPMsiR$_{6\text{-}8,}$ are unable to restore virus production in NPM1 depleted cells.

4.2.2 Depletion of cellular NPM1 results in the diffuse cytoplasmic distribution of select Rev-dependent viral RNAs

My results demonstrating depletion of NPM1 expression substantially reduced virus production in HeLa cells and MDM supports a role for NPM1 in viral replication (Figure 4-1 and 4-2). However its precise role in Rev function during viral replication has yet to be identified. Although my previous work has suggested NPM1 does not have a role in Rev-mediated vRNA export function (Chapter 3), it remained unclear whether NPM1 has a role in the intron-containing vRNA localization and utilization functions of

Rev. To examine whether NPM1 takes part in these Rev functions during viral replication, I analyzed the intracellular distribution of intron-containing vRNAs post-infection, using coupled RNA FISH and indirect immunofluorescence. HeLa cells were Mock transfected or transfected with 5 nM of siANC or siNPM1. After 34hrs of siRNA treatment, cells were trypsinized, counted and re-seeded onto cover slips in triplicate for analysis by fluorescence microscopy while the remaining cells were re-seeded onto culture dishes without cover slips for analysis by Western blotting. The next day, cells were infected with VSV-G pseudotyped NL4-3 virus (MOI= 4) then fixed or harvested 24hrs later. A Cy3-conjugated RRE-specific probe was used to detect intron-containing vRNA while an anti-NPM1 primary antibody followed by an AF-488 conjugated secondary antibody was used to detect NPM1. Treatment with reagent or siANC did not effect NPM1 intracellular localization or expression (Figure 4-6A and B). In these treated cells, cellular NPM1 was observed predominately concentrated in the GC of the nucleolus with some nuclear localization, consistent with my previous observations (Figure 3-6A, B and Figure 3-7A, B). NPM1 expression was markedly reduced in HeLa cells treated with siNPM1, whereas similar basal levels of NPM1 were observed in the Mock and siANC-treated cells (Figure 4-6). In the infected Mock and siANC treated cells, I observed two patterns of intron-containing vRNA localization. The most distinct localization was abundant, large punctae accumulating at, and radiating from, the perinuclear region of the cells (Figure 4-6B, first and second panel). In addition, a small fraction of the intron-containing vRNAs in these cells was observed to be distributed throughout the cytoplasm (Figure 4-6B, first and second panel). These findings are

consistent with previous reports for *gag* vRNA localization in HeLa and Cos cells (175, 177). However, a remarkable difference in intron-containing vRNA localization was observed in siNPM1 treated cells. Very few punctae were observed in these cells compared with Mock and siANC treated cells (Figure 4-6B). And the punctae detected appeared more intracellularly dispersed rather than accumulating at the perinuclear region (Figure 4-6B). The most striking observation in the siNPM1 treated cells was the widely dispersed cytoplasmic localization of the intron-containing vRNAs (Figure (4-6B, bottom panel). This observed difference of vRNA localization is unlikely attributed to a general affect of siNPM1 treatment on RNA localization. I have validated this experimental approach, showing NPM1-specific gene silencing does not affect cellular mRNA localization and/or expression in infected cells. Specifically, I observed that the ubiquitously expressed GAPDH RNA localization was not altered when NPM1 expression was depleted (Figure A1-6). It is also important to note the absence of punctae detected in the Mock-treated uninfected cells, thus demonstrating the specificity of the probe (Figure 4-6A). Of importance, Western blot analysis revealed nearly identical intracellular Gag expression detected in infected cells depleted of NPM1 as Mock and siANC treated cells (Figure 4-6C). Collectively, these results support a role for NPM1 in the cytoplasmic localization of Rev-dependent vRNA.

To extend these results, I used a Cy3-conjugated *gag-pol* RNA-specific oligonucleotide probe to localize full-length vRNA in infected HeLa cells depleted of NPM1 expression (Figure 4-7B). Since the Cy3-conjugated RRE-specific probe detects transcripts containing the *cis*-acting element and my results in Mock and siANC treated

cells support previous studies that the observed localization of large punctae accumulate at, and radiate from, the perinuclear region in addition to localization throughout the cytoplasm (Figure 4-6B, first and second panel) (175, 177), then it would be likely that a *gag-pol* RNA-specific probe would yield a similar localization pattern as the RRE-specific probe (Figure 4-6). Therefore, HeLa cells were Mock or siRNA treated for 34hrs then trypsinized, counted and re-seeded in triplicate onto cover slips, while the remaining cells were re-seeded onto culture dishes without cover slips. The next day, cells were infected with VSV-G pseudotyped NL4-3 virus (MOI= 4) and harvested 24hrs later. Fixed cells on cover slips were analyzed by FISH using a *gag-pol* RNA-specific probe, followed by indirect immunofluorescence to detect NPM1, and visualized by fluorescent microscopy. Again, I observed the same nucleolar and nuclear localization for NPM1 in Mock and siANC treated cells and substantial diminution of NPM1 detected in siNPM1 treated cells as previously described (Figure 4-6, Figure 3-6A, B and Figure 3-7A, B). In addition, I observed a similar pattern of large *gag-pol* vRNA punctae accumulating around the perinuclear region and distributed outward throughout the cytoplasm in the Mock and siANC treated cells, which were detected using the RRE-specific probe (compare Figure 4-7B and Figure 4-6B first and second panels). The siNPM1 treated cells exhibited limited punctae and a diffuse cytoplasmic distribution of *gag-pol* vRNA, which was also observed using the RRE-specific probe (compare Figure 4-7B and Figure 4-B bottom panels). Moreover, no substantial difference in Gag protein expression was observed in NPM1 depleted cells (Figure 4-7C). Therefore, these results further support a role for NPM1 in the cytoplasmic localization of Rev-dependent vRNA. More

specifically, no quantitative difference in Gag protein expression was observed in cells depleted of NPM1. Cells depleted of NPM1 exhibit significant diminution in virus production and diffuse vRNA localization during viral replication, suggesting this difference could be due to mislocalization of gRNA and supporting a role for NPM1 in the utilization of Rev-dependent vRNA.

To further differentiate which class(es) of intron-containing vRNA were affected by NPM1 depletion, I examined the intracellular localization of *env* vRNA in siRNA treated cells. The *env* RNA-specific oligonucleotide probes were designed based on HIV-1 isolates complementarity to the *env* splice junction, and thus do not cross hybridize with sequences in *gag-pol* vRNA. HeLa cells were treated for 34 hrs with Mock, siANC or siNPM1 siRNA, then trypsinized, counted and re-seeded in triplicate onto cover slips. The remaining cells were re-seeded onto culture dishes without cover slips. The following day, cells were infected with VSV-G pseudotyped NL4-3 virus (MOI= 4) and harvested 24hrs post infection. Fixed cells on cover slips were analyzed by FISH using an *env* RNA-specific probe, followed by indirect immunofluorescence with an anti-NPM1 antibody and visualized by fluorescent microscopy. Cell lysates were prepared and analyzed by western blotting for the expression of NPM1, Gag and Actin. Interestingly, I observed no discernible difference in the intracellular localization of *env* vRNA in treated cells. Specifically, I observed bright, discrete, large punctae localized predominately at the cell periphery with some *env* vRNA accumulation also observed throughout the cytoplasm in Mock, siANC and siNPM1 treated cells (Figure 4-8B). In contrast, I observe a decreased level of NPM1 detection in the siNPM1 treated cells only,

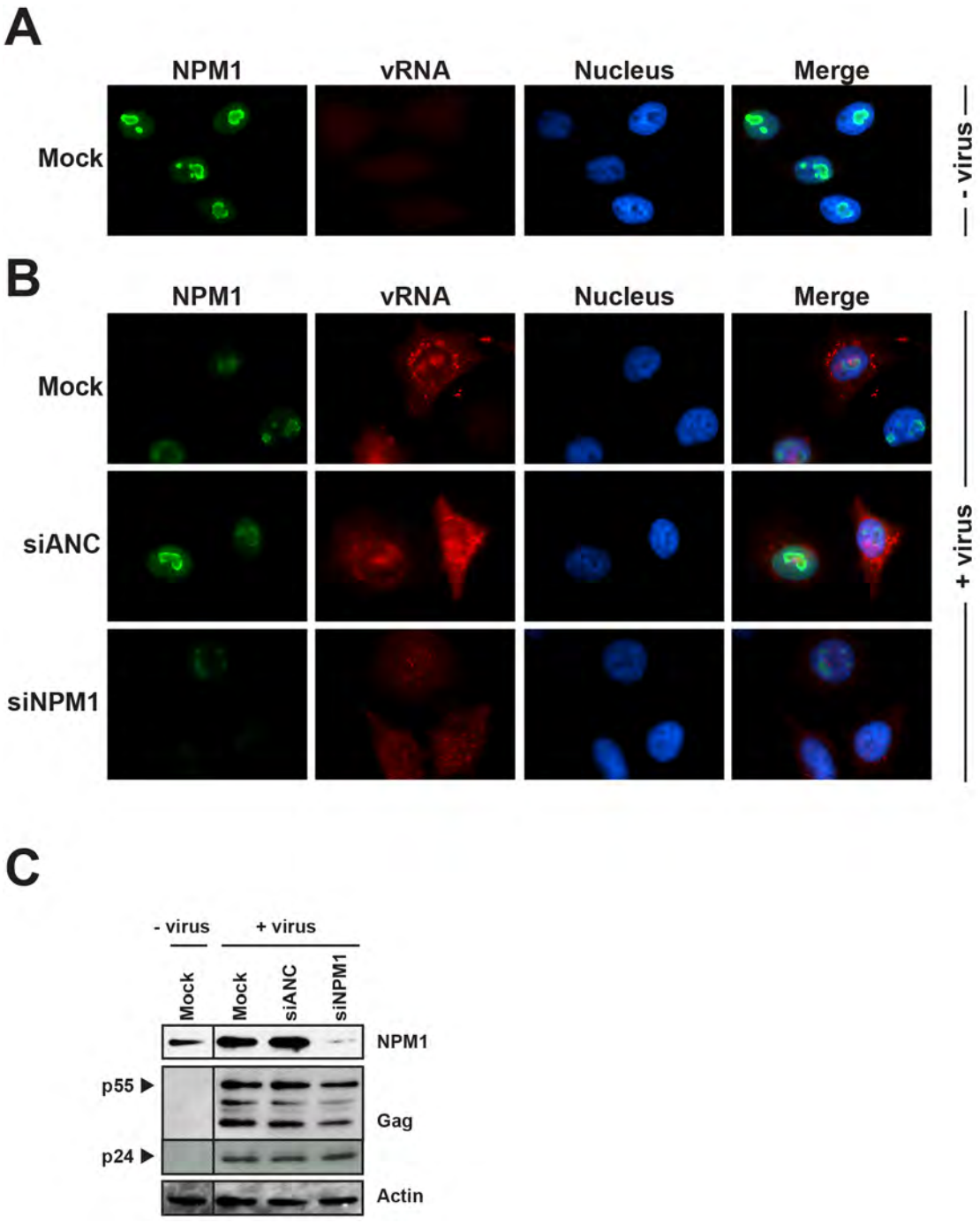


Figure 4-6. Depletion of NPM1 expression alters HIV-1 intron-containing vRNA cytoplasmic localization. (A, B) HeLa ells were transfected with reagent alone (Mock;

A, B top panel), siANC (B middle panel) or siNPM1 (B lower panel). At 48hrs post siRNA transfection, cells were uninfected (A) or infected with VSV-G pseudotyped NL4-3 virus at an MOI of 4 (B). At 24hrs post-infection, cells were fixed with 4% paraformaldehyde/5 mM $MgCl_2$/1X PBS and analyzed using coupled FISH- IF. A Cy3-conjugated RRE vRNA-specific probe was used to detect intron-containing vRNA (red). Immune detection of NPM1 (green) was performed using anti-NPM1 primary antibody and an AF-488 conjugated secondary antibody. The nucleus (blue) was detected by DAPI. Data shown is representative of multiple fields examined from three independent experiments. (C) HeLa cell lysates prepared from experiments in A and B were analyzed for NPM1 gene-specific knock-down and intracellular Gag expression by Western blotting using an anti-NPM1 antibody and anti-Gag antibody, respectively.

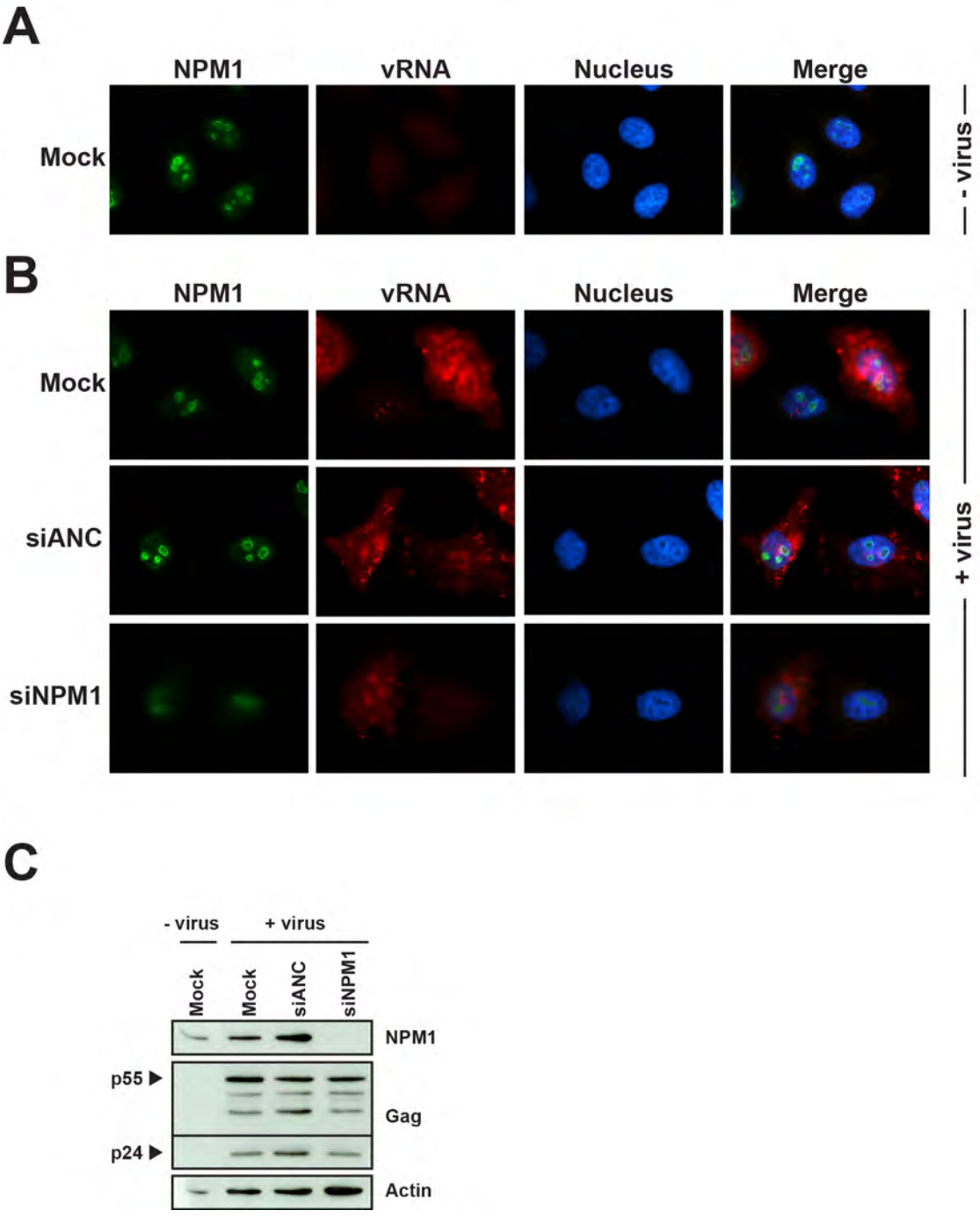


Figure 4-7. Depletion of NPM1 expression alters HIV-1 gag-pol vRNA cytoplasmic localization. (A, B) HeLa cells were transfected with reagent alone (Mock;

A, B top panel), siANC (B middle panel) or siNPM1 (B lower panel). At 48hrs post siRNA transfection, cells were uninfected (A) or infected with VSV-G pseudotyped NL4-3 virus at an MOI of 4 (B). At 24hrs post-infection, cells were fixed with 4% paraformaldehyde/5 mM $MgCl_2$/1X PBS and analyzed using coupled FISH- IF. A Cy3-conjugated *gag-pol* RNA-specific probe was used to detect genomic vRNA (red). Immune detection of NPM1 (green) was performed using anti-NPM1 primary antibody and an AF-488 conjugated secondary antibody. The nucleus (blue) was detected by DAPI. Data shown is representative of multiple fields examined from one experiment. (C) HeLa cell lysates prepared from experiments in A and B were analyzed for NPM1 gene-specific knock-down and intracellular Gag expression by Western blotting using an anti-NPM1 antibody and anti-Gag antibody, respectively.

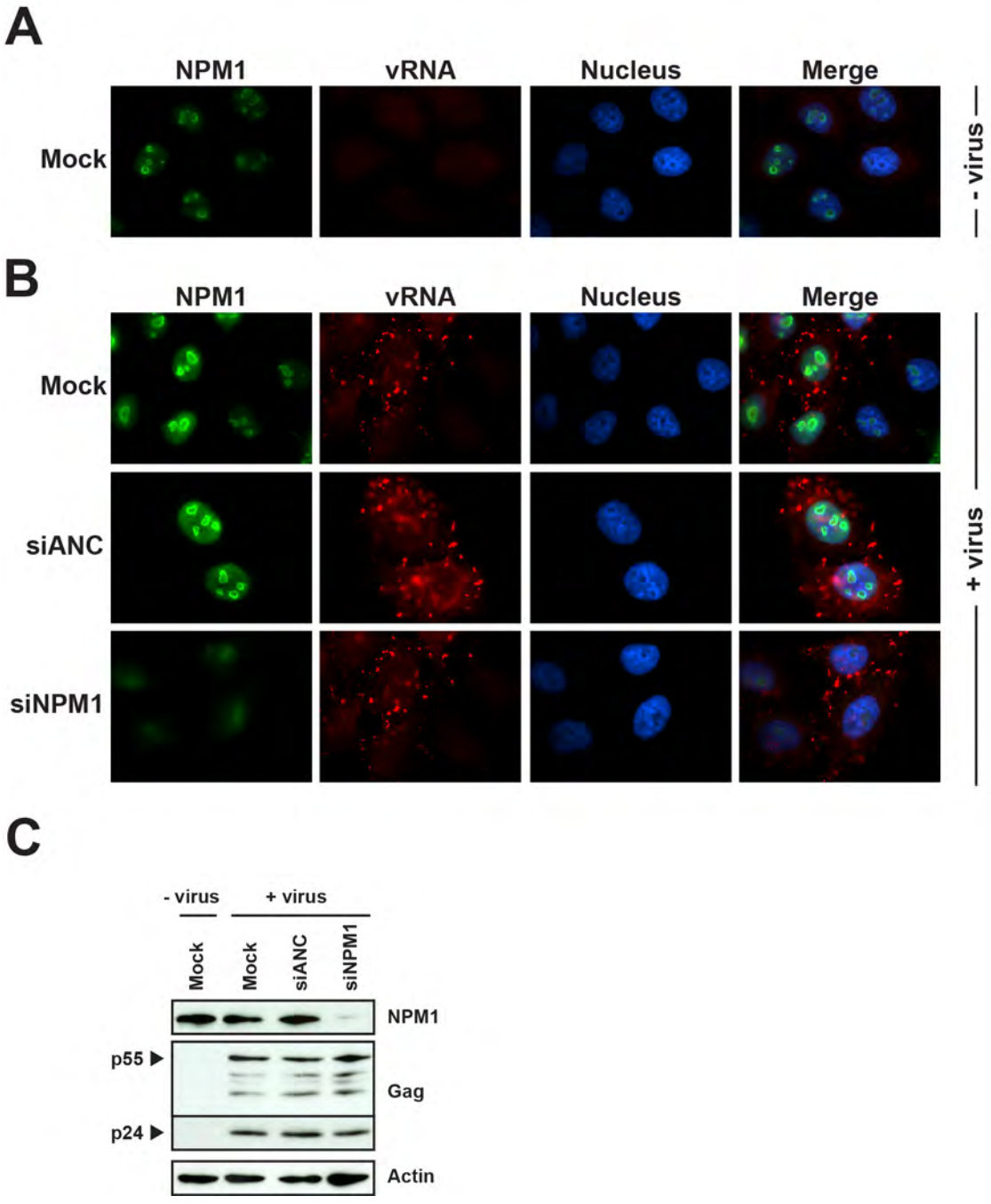


Figure 4-8. Depletion of NPM1 expression by RNAi has no discernable effect on HIV-1 env vRNA localization. (A, B) HeLa cells were transfected with reagent alone

(Mock; A, B top panel), siANC (B middle panel) or siNPM1 (B lower panel). At 48hrs post siRNA transfection, cells were uninfected (A) or infected with VSV-G pseudotyped NL4-3 virus at an MOI of 4 (B). At 24hrs post-infection, cells were fixed with 4% paraformaldehyde/5 mM $MgCl_2$/1X PBS and analyzed using coupled FISH- IF. A Cy3-conjugated *env* RNA-specific probe was used to detect envelope vRNA (red). Immune detection of NPM1 (green) was performed using anti-NPM1 primary antibody and an AF-488 conjugated secondary antibody. The nucleus (blue) was detected by DAPI. Data shown is representative of multiple fields examined from three independent experiments. (C) HeLa cell lysates prepared from experiments in A and B were analyzed for NPM1 gene-specific knock-down and intracellular Gag expression by Western blotting using an anti-NPM1 antibody and anti-Gag antibody, respectively.

indicating I achieved specific depletion of the protein (Figure 4-8B). Efficient depletion of NPM1 was confirmed by Western blotting (Figure 4-8C, top panel). Western blot analysis also showed no evident difference in intracellular Gag expression, consistent with my previous findings. Thus, these data, taken together with my other localization findings, strongly argues NPM1 affects the cytoplasmic localization and utilization of a specific subset of Rev-dependent vRNA, the *gag-pol* vRNA.

While I observed a marked reduction in virus production in cells treated with NPM1-specific siRNAs, it was unclear whether cells depleted of NPM1 support production of infectious HIV-1 particles. To investigate this possibility, HeLa cells were Mock transfected or transfected with 5 nM of siANC or 5 nM of siNPM1. After 48hrs of siRNA treatment, cells were infected with VSV-G NL4-3 virus (MOI = 4). Culture supernatants were collected 24- and 48hrs post infection to quantify virus production by RT enzyme activity assays (Figure 4-9A). To determine the infectivity of virus present in culture supernatants, I used a TZM-bl indicator cell-based assay that monitors Tat-dependent β-gal expression. TZM-bl cells were infected with culture supernatants containing equivalent RT units of virus cultures for 48hrs, then subsequently washed, fixed and assayed for β-gal enzymatic activity. RT enzyme activity assay analysis revealed a significant reduction in virus production in the presence of siNPM1 at both time points examined (Figure 4-9A). Western blot analyses show efficient depletion of NPM1 expression in only siNPM1 treated cells, while Gag expression levels remained unchanged when compared with Mock or siANC treated cells (Figure 4-9B). These findings confirm results I have previously reported (Figure 4-1, Figure A1-3, Figure 4-5).

Although I do see a significant diminution in the level of virus produced in siNPM1 treated cells, the corresponding TZM-bl virus infectivity assay data indicate the virus particles are as infectious as those produced in Mock and siANC treated cells (Figure 4-9C, B). Thus, production of infectious HIV-1 particles is unaltered in the absence of NPM1 expression.

In the results presented, I used viral RT enzyme activity assay analysis to quantify virus production. Nevertheless, Gag proteins are necessary and sufficient for the assembly and release of virus-like particles (VLPs) (112, 116). To examine whether siNPM1 depletion has an effect on Gag release, I utilized a standard HIV-1 p24 ELISA to quantify p24 concentration in siRNA treated MDM culture supernatants at 24- and 48hrs post infection. The culture supernatants quantified for p24 production were those collected and assayed for RT enzyme activity reported in Figure 4-2. Interestingly, my p24 ELISA results revealed a minor diminution in p24 production in siNPM1 treated cells at 24hrs post infection, where an 88% p24 production was observed (Figure 4-10). However, at 48hrs post infection p24 production was reduced further to 57%. Multiple student T-test analysis determined the decrease in p24 production in the siNPM1 treated cells to be non significant at both time points (P= 0.22317, P= 0.06207). Nonetheless, these findings, together with the observed decrease in virus production in siNPM1 treated cells quantified by RT enzyme activity assays, nearly unchanged expression of intracellular Gag protein as well as dispersed localization of *gag-pol* vRNA further support a role for NPM1 in the localization and utilization of *gag-pol* vRNA.

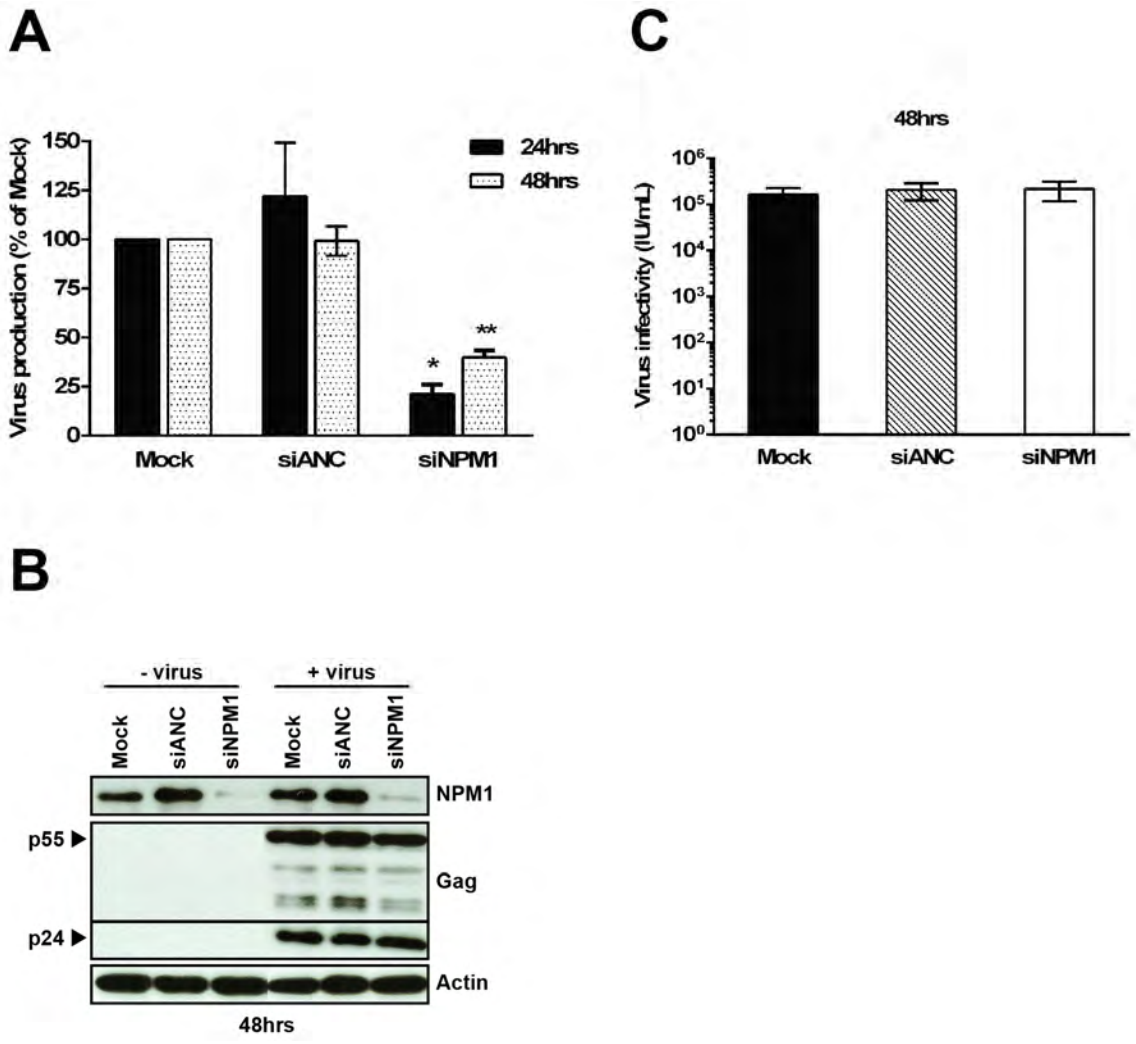


Figure 4-9. Depletion of NPM1 expression by RNAi has no discernable effect on HIV-1 infectivity. HeLa cells were transfected with reagent alone (Mock), 5 nM of siANC or 5 nM of siNPM1. At 48hrs post siRNA transfection, cells were uninfected or infected with VSV-G pseudotyped NL4-3 virus at an MOI of 4. (A) Virus production was determined by quantifying viral RT enzyme activity of culture supernatants 24- and 48hrs post infection as described in Material and Methods. Bars represent the total mean

percent of virus production from two independent experiments, one performed in duplicate ± SEM. Multiple student T-test analysis determined significant P values at 24hrs (*, P≤ 0.05) and 48hrs (**, P≤ 0.01). (B) Cell lysates were analyzed for NPM1 gene-specific knock-down and intracellular Gag expression by Western blotting 48hrs post infection with an anti-NPM1 and anti-Gag antibody, respectively. Data shown is representative of cell lysates prepared from experiments in A. (C) The infectivity of virus present in culture supernatants collected 48hrs post infection was determined using TZM-bl indicator cells and monitoring β-gal expression. Aliquots of supernatants containing equivalent RT activity units were serially diluted 10-fold in antibiotic-free media. Duplicate cell monolayers were inoculated with the diluted virus cultures. At 48hrs post infection, cells were washed, fixed and assayed for β-gal enzymatic activity as described in Materials and Methods. Bars represent the total mean virus infectivity of culture supernatants collected from experiments in A ± SEM. A random-block one way ANOVA analysis determined non significant P values for virus infectivity (P= 0.2748).

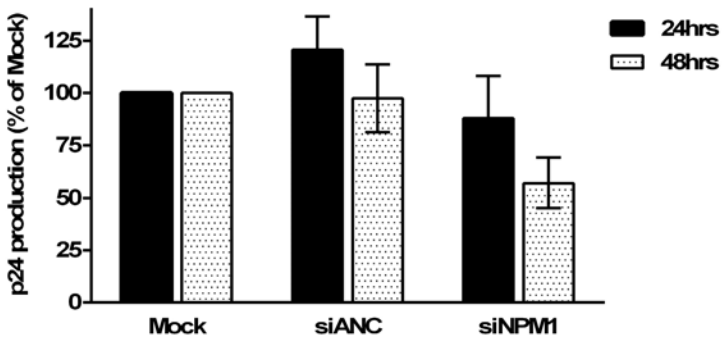


Figure 4-10. Depletion of NPM1 expression by RNAi has a marginal effect on p24 production in primary human macrophages. Culture supernatants collected from experiments reported in Figure 4-2 were analyzed for HIV-1 Gag p24 production by standard HIV-1 p24 ELISA 48hrs post infection as described in Material and Methods. Bars represent the total mean percent of p24 production from nine independent experiments ± SEM. Multiple student T-test analysis determined non significant P values at 24hrs (P= 0.22317) and 48hrs (P= 0.06207).

4.3 Discussion

In this study, I examined whether the previously identified Rev-cofactor, NPM1 has a role in Rev function and/or post-transcriptional events during viral replication. Although a number of studies have reported NPM1 to interact with Rev and suggested a role for NPM1 in Rev function, these reports were unable to establish a functional link between NPM1 and Rev *in vivo* (100, 304, 305, 306). Notably, no studies to date have examined whether NPM1 participates in Rev-mediated export in the context of viral replication. Furthermore, it is unknown whether NPM1 has a role in other identified Rev functions, such as vRNA localization, utilization and translation (reviewed in (138, 141, 174, 299, 301)) or whether NPM1 affects virus production post-transcriptionally. Here, I am the first to report using an RNAi-based strategy to examine NPM1 involvement in Rev-dependent vRNA post-transcriptional events during viral replication in cell lines and primary human macrophages. I am able to achieve specific, efficient siRNA-mediated depletion of cellular NPM1 in HeLa cells and MDM (Figure 4-1B and Figure 4-2B and Figure A1-1 through Figure A1-4). Following siNPM1 treatment, I show a marked, statistically significant reduction of virus production in HeLa cells and MDM infected with VSV-G pseudotyped wildtype virus at 24- and 48hrs post infection (Figure 4-1A and Figure 4-2B). Importantly, these results are further substantiated in NPM1 depleted HeLa cells transiently transfected with wildtype virus as well as NPM1 depleted HeLa cells infected with VSV-G pseudotyped wildtype virus at a lower MOI (Figure A1-2 and A1-3). While I am the first to report a role for NPM1 during HIV-1 infection, NPM1 has been implicated in post-transcriptional events of other retroviruses. Caporale *et al.*

identified the 80 amino acid signal peptide of the simple Jaagsiekte Sheep Retrovirus (JSRV) Envelope glycoprotein (Env) as a posttranscriptional regulator of viral gene expression, functioning through similar mechanisms as HIV-1 Rev (48). Using transient co-transfection experiments in 293T and Cos cells, they demonstrate the JSRV signal peptide (JSE-SP) co-localizes with NPM1 in the nucleolus, enhances the nuclear export of unspliced vRNA and increases virus production (48). Importantly, when increasing concentrations of dominant negative NPM1 is co-transfected with JSE-SP, a dose-dependent decrease of virus production is observed. They also showed JSE-SP interacts with NPM1 in the nucleus and the cytoplasm, and surprisingly, associated together with membrane/organelles (48). Thus, these data suggest JSE-SP function is dependent on NPM1 activity. Of significance, in these studies they utilized a HIV-1 *gag-pol* expression plasmid co-transfected with Rev and dominant negative NPM1, which also demonstrated a decrease in virus production comparable to what I observe in NPM1 depleted, infected HeLa cells and MDM. Taken together, these studies substantiate a NPM1 role in HIV-1 replication. Collectively, these findings provide complimentary evidence NPM1 is required for HIV-1 replication.

To further demonstrate a role for NPM1 in HIV-1 replication, I examined whether virus production could be restored in cells depleted of NPM1 expression. I developed stably transduced cell lines expressing siRNA-resistant GFP-tagged variants of cellular NPM1 using the previously characterized wildtype pGFP-NPM1 to generate the mutants (Figure 4-3, Figure 4-4, Figure 4-5 and Figure A1-5) (97, 98). However, despite being resistant to siNPM1 treatment, GFP-NPMsiR$_{2-4}$ and GFP-NPMsiR$_{6-8}$ stable cell lines

were unable to restore wildtype levels of virus production in NPM1 depleted cells (Figure 4-5). There are several possible explanations as to why these stable cell lines were unable to augment virus production. For example, the correctly folded three-dimensional structures of GFP-NPMsiR fusion proteins may differ considerably from that of native NPM1, rendering them functionally inactive. My Western blotting results show the anti-GFP antibody is able to detect a greater amount of GFP-NPMsiR protein than the anti-NPM1 antibody, supporting this probability. This insight is further corroborated by the inability of the anti-NPM1 antibody to detect GFP-NPMsi$R_{2\text{-}3}$ even though there are no amino acid differences from wildtype NPM1 (Figure 2-1). It is necessary to note that all mutations generated within each GFP-NPMsiR are silent, meaning no amino acid residues were changed (Figure 2-1). Alternative folding of the GFP-NPMsiR proteins could occlude access to functional domains and protein: protein and/or protein: nucleic acid interactions necessary for virus production. Furthermore, protein misfolding could hinder the ability of NPM1 to be modified by phosphorylation, acetylation, ubiquitylation, and SUMOylation, all of which have been indicated to regulate different aspects of function, localization and oligomerization (117, 205, 241). The ability for NPM1 to properly oligomerize via the core domain has been shown to be important for molecular chaperone ability and facilitate subcellular localization (117, 205, 241, 244, 265). Another contributing factor may be that fluorescent fusion proteins have a weak tendency to oligomerize and when these fusion proteins are targeted to specific cellular compartments, local accumulation can promote dimerization (272, 340). Since GFP-NPM1siR proteins concentrate in the nucleoli, the propensity of GFP to self-dimerize

may supersede NPM1 core domain-driven oligomerization and thus affect function (Figure 4-3). Lastly, my characterization of the GFP-NPMsiR cell lines was limited to examining fusion protein localization, the ability of these cells to support virus replication and confirming resistance to siNPM1 treatment. Thus, investigating the functional properties of the expressed GFP-NPMsiR proteins is warranted. Such functional analysis of the GFP-NPMsiR proteins could include testing the ability of these proteins to bind nucleic acid, decondense sperm chromatin, transfer nucleosomes to naked DNA and assist in renaturning chemically denatured proteins, *in vitro* (149, 151, 242, 243, 307, 326)). Moreover, construction, expression and analysis of alternative NPM1 siRNA-resistant fusion proteins expression constructs, such as polyhistidine or c-Myc tags, may address this unresolved issue.

Interestingly, the reduced level of virus production observed in HeLa cells and MDM was not associated with a considerable diminution of intracellular Gag expression in these cells, indicating the reduction in virus is not due to insufficient Gag expression. This data is consistent with my findings that depletion of NPM1 expression does not affect Rev-dependent vRNA export activity and extends my findings that NPM1 is not required for Rev-mediated vRNA export during replication (Chapter 3, Figure 3-8). Moreover, these data suggest the observed siNPM1-mediated reduction in virus production is also not due a virus assembly or budding defect. HIV-1 Gag is synthesized on cytosolic polysomes as a 55 kD polyprotein precursor (p55) comprised of four domains: matrix (MA/p17), capsid (CA/p24), nucleocapsid (NC/p7) and p6 with two spacer peptides, SP1, located between CA and NC, and SP2, found between NC and p6.

These domains are separated by five flexible linker regions that contain sites cleaved by PR during virion maturation to liberate the processed forms of the proteins (111, 112, 300). Research investigating HIV-1 virus assembly and budding describe Gag processing defects associated with disruptions of these processes, such that accumulation of cell-associated Gag processing intermediates can be observed (125, 130, 131, 218, 257, 297, 321). The Gag cell-associated processing products detected by Western blotting with the anti-Gag antibody reveal no significant differences between siNPM1, Mock and siANC treated cells, therefore indicating siNPM1-mediated reduction in virus production is not due to a virus assembly or budding defect (Figure 4-1B, Figure 4-2B, Figure A1-2B and Figure A1-3B).

Examination of Rev-dependent vRNA localization in the absence or presence of siNPM1 treatment reveals significant differences in intracellular localization of certain Rev-dependent vRNAs when NPM1 is depleted (Figure 4-6 through Figure 4-8). In the infected Mock and siANC treated cells, I observed two patterns of vRNA localization using the RRE RNA-specific and *gag-pol* RNA-specific probes; large, abundant punctae accumulating at and radiating from the perinuclear region and a small fraction distributed throughout the cytoplasm (Figure 4-6B and Figure 4-7B). These findings are consistent with previous reports of *gag* vRNA localization in HeLa and Cos cells (175, 177). Remarkably, the RRE-containing and *gag-pol* vRNA infected siNPM1 treated cells localize to limited number of punctae and display diffuse cytoplasmic distribution, implicating NPM1 in the cytoplasmic localization of Rev-dependent vRNA (Figure 4-6B and Figure 4-7B). Analysis of *env*-specific vRNA localization further defined the role for

NPM1 vRNA localization. Because the *env* vRNA localization was not altered when NPM1 was depleted, clearly suggests a role for NPM1 in the cytoplasmic localization of *gag-pol* vRNA (Figure 4-8B). When considered with the demonstrated reduction in virus production in HeLa cells and MDM following siNPM1 treatment, these findings extends my model to include a role for NPM1 in the cytoplasmic utilization of *gag-pol* vRNA.

Identification of NPM1 as a modulator of *gag-pol* vRNA localization and/or utilization is not speculative. A number of host cellular factors have been identified to associate with Rev-dependent vRNPs and facilitate post-transcriptional functions (reviewed in (65, 141, 224)). The *gag-pol* vRNA serves as an mRNA template for Gag and Gag-Pol polyprotein synthesis as well as the genome that is packaged into nascent virions (111, 112, 300). Research to date has not found HIV-1 to have distinct pools of *gag-pol* vRNA to select transcripts for translation or packaging (42). However, numerous groups have implicated the composition of the vRNP as a mediator for the localization and/or utilization of *gag-pol* vRNA, which can be determined in the nucleus, at the nuclear pore or in the cytoplasm (2, 19, 58, 59, 60, 65, 71, 147, 206, 209, 223, 228, 232, 233, 292). The cellular protein Sam68 (Src-associated during mitosis 68 kD) has been suggested to enhance 3' end processing of unspliced vRNA and may mark the gRNA in the nucleus to promote cytoplasmic utilization (71, 223). DEAD-box helicases DDX1 and DDX3 have been reported to be involved in Rev-mediated transport and modulate Rev subcellular localization (99, 336), while DDX24 has been shown to promote gRNA packaging (209). Moreover, hnRNP A2 and Staufen have been shown to associate with the genomic RNP, promoting gRNA trafficking and packaging (2, 19, 58,

59, 60, 228). Intriguingly, NPM1 has been implicated in mRNA 3' end processing and identified as a component of an RNP containing DDX1, CRM1, Nup62, Nucleolin and hnRNPA1, which are also cellular factors that maintain post-transcriptional roles in HIV-1 replication and/or Rev function (65, 141, 154, 210, 224, 233, 277, 317). These findings reveal how intimately coupled NPM1 is to various Rev-dependent vRNA post-transcriptional processes.

In conclusion, my data strongly supports a role for NPM1 in the localization and utilization of gRNA during HIV-1 replication. To date, no studies have examined NPM1 and Rev-dependent vRNA in the absence or presence of Rev *in vivo* or during infection. Moreover, none have examined NPM1 in the context of full-length RNA or even the complete RRE. My findings that depletion of cellular NPM1 has an adverse effect on HIV-1 production but not Gag expression and can alter the intracellular distribution of *gag-pol* vRNA support a model in which NPM1 plays an active role in the cytoplasmic localization utilization of *gag-pol* vRNA and possibly, gRNA packaging. Quantitative analysis of gRNA in nascent particles produced in the absence or presence of cellular NPM1 would provide key insights into this possibility. Whether the functional involvement of NPM1 in *gag-pol* vRNA localization and utilization occurs in the cytoplasm, nucleus, or nucleolus remains to be determined. Moreover, the molecular mechanism by which NPM1 associates with the *gag-pol* vRNA/RNP (i.e.protein: protein or protein: vRNA) warrants further investigation.

CHAPTER V: DISCUSSION AND FUTURE DIRECTIONS

CHAPTER V: DISCUSSION AND FUTURE DIRECTIONS

5.1 Summary

The results from this thesis present novel findings to implicate a nucleolar and NPM1 involvement in distinct aspects of intron-containing vRNA export and post-transcriptional regulation. Using coupled RNA fluorescent *in situ* hybridization and immunofluorescent analysis, I demonstrate that intron-containing vRNA localize as intense, discrete foci in the nucleus. I show for the first time localization of the intron-containing vRNA foci that are detected as early as 6hrs post infection and remain as discrete, bright foci up to 24hrs, with a saturation point of 30hrs post infection. Importantly, I demonstrate that cells infected with wild type virus, Rev-deficient virus or viruses containing a mutated RRE enabling higher Rev binding affinity, all display distinct intron-containing vRNA foci that localize coincident to the nucleolus independent of Rev expression. My findings of intron-containing vRNA foci localizing to similar discrete nuclear regions support a model that involves cellular factors mediating intron-containing vRNA localization rather than the viral protein Rev. Furthermore, localization of the intron-containing vRNAs coincident to the nucleolus, a domain found to be enriched in Rev and Rev binding partners, provides evidence for the nucleolus as a site for intron-containing vRNA localization during Rev-mediated vRNA export. I further extend a role for the nucleolus to post-transcriptional events of intron-containing vRNA by investigating the functional involvement of the previously identified Rev co-factor and nucleolar protein, NPM1 in Rev function and viral replication. I report the results of the first RNAi-based strategy to examine NPM1 involvement in Rev

nucleolar localization and Rev-mediated vRNA export *in vivo*. I show efficient, specific depletion of NPM1 expression in siNPM1 treated cells without toxicity. Significantly, I demonstrate the nucleolar localization of Rev does not change when NPM1 expression is depleted, indicating NPM1 does not have a role in the nucleolar localization of Rev, thus supporting the premise other cellular factors mediate this localization. I used a CAT reporter co-transfection assay to monitor Rev export activity in the presence or depletion of cellular NPM1. I demonstrate that despite efficient cellular NPM1 depletion, no significant inhibition of CAT activity is observed, indicating NPM1 does not participate in Rev vRNA export. These results are further established and extended in my viral replication studies. I report the first experiments to use a viral infection and an RNAi-based strategy to examine NPM1 involvement in Rev function and viral replication. I demonstrate that NPM1 depletion in cell lines and primary human macrophages confers a significant reduction in virus production. I also show this reduction is not associated with a diminution of intracellular Gag expression, indicating that reduction in virus production is not due to insufficient Gag expression. Through examination of the localization of Rev-dependent vRNA in the absence or presence of siNPM1 treatment, I reveal significant differences in specific Rev-dependent vRNAs when NPM1 is depleted. Instead of large, abundant vRNA punctae accumulating at and radiating from the perinuclear region with a small fraction of vRNA distributed throughout the cytoplasm as seen in reagent and siANC treated cells, the *gag-pol* RNA localization in siNPM1 treated cells have limited punctae and a diffuse cytoplasmic distribution. In contrast, the *env* vRNA localization is unaltered when NPM1 is depleted, thus supporting a role for NPM1

in the cytoplasmic localization of *gag-pol* vRNA. These data, taken together with my findings of reduced virus production in HeLa and MDM siNPM1 treated cells, extends this model to include a role for NPM1 in the cytoplasmic utilization of *gag-pol* vRNA. In addition, I show the siNPM1 treated cells release similarly infectious virions as control treated cells and p24 production is not significantly reduced. These data strongly support a model where NPM1 modulates gRNA localization and utilization during HIV-1 replication. Collectively, my findings provide novel insight for a functional role of the nucleolus and NPM1 in HIV-1 replication, which enhances our current understanding of HIV-1 biology.

5.2 The Nucleolus and Intron-containing vRNA Export

Current research supports a model for nucleolar localization of vRNA during HIV-1 replication. Both vRNA and HIV-1 Rev have been shown to localize to the nucleolus and the nucleolus has been implicated as a site for Rev-mediated export complex formation (41, 46, 64, 75, 77, 204, 225, 227, 229, 260, 271, 274, 294, 346). However, the involvement of the nucleolus for vRNA localization prior to export has remained inconclusive. Several studies examining intron-containing vRNA localization using electron microscopy *in situ* hybridization report conflicting evidence; some report detection of intron-containing vRNA in the nucleolus while others do not (46, 64, 274). Furthermore, research has yet to distinguish the class(es) of vRNAs that may localize to the nucleolus or establish how their nucleolar localization could be mediated. In this thesis, I am the first to report intron-containing vRNA localization in the presence or

absence of Rev during a viral infection and detect the regions of the nucleolus as a localization reference. Using coupled fluorescent *in situ* hybridization and immunofluorescent analysis, I demonstrate cells infected with wild type virus, Rev-deficient virus or viruses containing a mutated RRE enabling higher Rev binding affinity, all display distinct intron-containing vRNA foci that localize coincident to the granular component of the nucleolus, independent of Rev expression. These findings add support to a model for intron-containing vRNA nucleolar localization prior to export. Surprisingly, however, these findings also suggest factor(s) other than Rev likely mediate intron-containing vRNA nucleolar localization. Collectively, my results presented in this thesis open exciting avenues to explore, to confirm intron-containing vRNA nucleolar localization and to delineate how this localization may be mediated.

It is important to consider the possibility there is no nucleolar localization step of intron-containing vRNA prior to export, or is a dead-end for the intron-containing vRNA once in the nucleolus. However, there are two key findings that strongly argue against these prospects in addition to the research presented and discussed in this thesis. First, Michienzi *et al.* demonstrate that lymphocytes stably transduced with a nucleolar localized hammerhead ribozyme designed to site-specifically cleave a conserved region within all HIV-1 RNA transcripts inhibits HIV-1 virus production for over 24 days, the length of the study (225). Second is the report that demonstrating a nucleolar localized Rev binding peptide is able to inhibit HIV-1 replication by sequestering Rev function for its export only (226). Together, these data strongly argue that intron-containing vRNA localize to the nucleolus for export.

There are numerous experiments that would further elucidate bone fide nucleolar localization for intron-containing vRNA during viral replication. As discussed in Chapter 3, electron microscopy *in situ* hybridization offers the ultrastructural resolution required for specific nucleolar localization. Using this technique to compare intron-containing vRNA localization during infection with my wild type, Rev-deficient or enhanced RRE-containing virus would be the first report of a direct evaluation for vRNA localization and Rev dependence during an infection. Confocal or deconvolution microscopy to analyze intron-containing vRNA localization would compliment the electron microscopy *in situ* hybridization experiments and provide greater serial spatial depth resolution than the wide-field fluorescence microscopy analysis presented in this thesis. Moreover, examination of Rev-independent vRNA localization by electron and/or fluorescence *in situ* hybridization is necessary.

I present evidence in this thesis to support intron-containing vRNA coincident nucleolar localization is Rev independent. To address how intron-containing vRNAs localize to the nucleolus, localization of cellular proteins identified to interact with intron-containing vRNA detected by IF while vRNA localization detected by FISH in the absence and presence of viral infection is needed. Proteins of interest include PSF, Matrin 3, DDX1 and hnRNP A1, all which have been previously shown to interact with intron-containing vRNA (80, 99, 185, 186, 232, 273, 286, 310, 333, 334, 347). Moreover, Rev localization should also be examined. Investigating the localization of these proteins and intron-containing vRNA in a time course study may also provide insight into the temporal aspect of nucleolar localization. Certainly, co-localization of

cellular proteins and intron-containing vRNA do not provide functional analysis, but can narrow the candidate protein pool for further analysis. Additionally, utilizing fluorescence resonance energy transfer (FRET) and fluorescence recovery after photobleaching (FRAP) methodologies to investigate possible cellular co-factors of Rev in the presence or absence of intron-containing vRNA could provide a live cell imaging approach to examine cellular and viral protein interactions and localizations during export. This method has been reported by the Pavlakis group to examine Rev multimerization and CRM1 cargo interactions in the nucleolus as well as the Parent group examining Rous Sarcoma virus (RSV) viral protein localization in the presence and absence of vRNA (23, 77, 78, 207).

An additional approach that should be employed is the isolation and examination of nucleoli for protein and vRNA content. The Lamond laboratory has established systematic protocols for isolation, RNA extraction and proteomic analysis of nucleoli for various cell types (6, 7, 189, 191, 248). Jarboui *et al.* adapted the Lamond protocol to report the first nucleolar proteomic profile of stably transduced HIV-1 Tat expressing Jurkat cells (169). To date, examination of proteomic profiles of isolated nucleoli in response to Rev expression has not been investigated. Of note, no reports have examined the nucleolar profiles (protein and/or vRNA) in the presence of vRNA or during a viral infection. Therefore, using the viral constructs I have developed in a similar system may provide new insight for a mechanism of HIV-1 vRNA nucleolar localization.

5.3 NPM1 and HIV-1 Rev: A New Model

NPM1 has been demonstrated to co-localize with Rev and has been suggested to be the cellular factor mediating Rev nuclear import and/or nucleolar localization (91, 100, 151, 229, 230, 304, 306). Research has shown that NPM1 binds Rev (100, 304, 305, 306), stimulates the nuclear import of Rev *in vitro* (306) and may be a component of the Rev export complex (204). Nonetheless, no research to date has demonstrated NPM1 is responsible for Rev import and/or nucleolar localization *in vivo* or has established a functional link between a NPM1:Rev interaction, nucleolar localization and Rev function. Therefore, results reported in this thesis are the first to examine NPM1 involvement in Rev nucleolar localization and Rev-mediated vRNA export *in vivo*. I report that despite almost complete reduction in intracellular NPM1 by siRNA treatment Rev retained its predominant nucleolar localization and Rev-mediated export function, thus supporting an alternative, NPM1 exclusion model for Rev nucleolar localization and export ability.

Surprisingly, it has been widely accepted that NPM1 mediates nucleolar localization of Rev despite the lack of *in vivo* evidence to support this premise (152, 204, 245, 294, 327). This hypothesis has been conveniently incorporated into several models for Rev function yet no studies have specifically examined NPM1 role in Rev function in the presence of full length vRNA, or even the entire RRE, *in vitro* or *in vivo* (Figure 5-1A) (100, 151, 241, 245, 304, 305, 306). Despite cell-free evidence, my cell-based results disagree with a NPM1 involvement in Rev nucleolar localization or Rev-mediated vRNA export (Figure 5-1B) (100, 304, 305, 306). For the Rev localization studies presented in this thesis, I used a GFP-fusion Rev protein demonstrated to localize

predominantly to the nucleolus (294). As previously noted, fluorescent fusion proteins have a weak tendency to oligomerize and when these are targeted to specific cellular compartments, local accumulation can promote dimerization (272, 340). Although this Rev-GFP expression construct has been widely used without experimental discrepancies, examination of Rev localization using different fusion protein tags in the absence or presence of NPM1 expression would complement the findings presented here. Moreover, including Rev localization experiments in the presence or absence of vRNA are also needed to provide further evidence as to whether NPM1 has a role in Rev nucleolar localization. An additional experiment would be to express a dominant negative NPM1 that does not localize to the nucleolus, such as the previously characterized GFP-NPM1MutA, to see whether Rev localization is altered (97, 98). This mutant could also be employed in the CAT reporter co-transfection assay to monitor the affect on Rev export activity (156, 157). Moreover, disruption of the identified Rev binding domains on an exogenously expressed NPM1 protein to monitor Rev localization could also clarify the reported difference in NPM1 mediating Rev localization and function (151, 305).

Research has suggested other factors may mediate Rev nucleolar localization. Early studies proposed Rev could accumulate in the nucleolus because of the high RNA content present and the fact that it has been shown to bind 5S rRNA (75, 188). However, upon further investigation, it is more widely acknowledged that cellular factors are likely to mediate the nucleolar localization. For example, several DEAD-box RNA helicases have been implicated, including DDX1 (99, 273, 333). Research described by Lin *et al.*

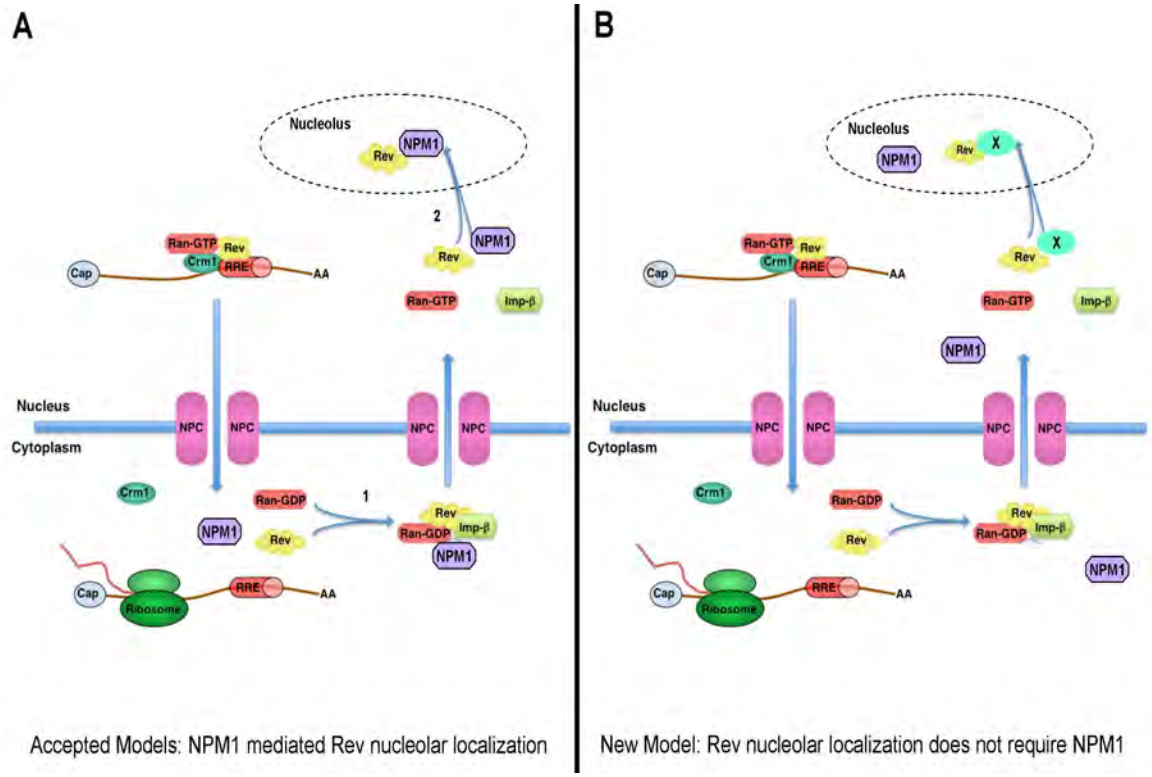


Figure 5-1. HIV-1 Rev nucleolar localization. (A) Two currently accepted models for HIV-1 Rev nucleolar localization. Research data has proposed NPM1 mediates the import of Rev from the cytoplasm to the nucleous, either as part of an import complex, or as the sole factor responsible (Model 1). While Model 2 purports a nuclear interaction of NPM1 and Rev to mediate Rev nucleolar localization. Interaction of Rev with NPM1 has been shown to be a short sequence in the NLS, while NPM1 interacts with Rev through a region in the N terminal. (B) Proposed model: HIV-1 Rev nucleolar localization does not require NPM1. Rev import complex does not require NPM1 and Rev nucleolar localization is not mediated by NPM1. Rev nucleolar localization is most likely mediated through interaction(s) with undetermined factor(s) in the nucleus (shown as X). *Adapted from Massimo Caputi. RNA Processing (2011).*

suggest a common factor between NPM1 and Rev could link their association in the nucleolus (204) One possible linker could be DDX1, since NPM1 has also been shown to be a part of a RNP with DDX1 and other cellular factors described to be a part of Rev-mediated vRNA transport and/or expression (210). However, these possibilities remain to be determined. FRET and FRAP methodologies to examine cellular factors that may mediate Rev nucleolar localization could provide this insight. Understanding the protein(s) and/or mechanism that mediate Rev nucleolar localization could elucidate whether the localization is necessary for Rev functions or Rev-dependent vRNA processing and/or export. Therefore, the research presented in this thesis is a step towards unraveling the complexity of HIV-1 vRNA export and expression.

5.4 NPM1 as a Modulator of *gag-pol* vRNA Localization and Utilization

Research has shown HIV-1 Rev not only mediates the nucleocytoplasmic export of the intron-containing vRNAs, but also has a functional role in the localization and utilization of Rev-dependent vRNAs (reviewed in (138, 141, 174, 299, 301)). Moreover, recent studies indicate Rev function extends to include the enhancement of gRNA encapsidation (26, 35, 119, 133, 134, 136, 208). NPM1 has also been identified to interact with Rev and shown to be a Rev co-factor (100, 304, 305, 306). However, no studies to date have examined whether NPM1 participates in Rev-mediated export in the context of viral replication. Furthermore, it is unknown whether NPM1 has a role in other identified Rev functions or whether NPM1 affects virus production post-transcriptionally. In this thesis, I am the first to report NPM1 involvement in Rev-

dependent vRNA post-transcriptional events during viral replication. My findings that depletion of cellular NPM1 inhibits HIV-1 production, determined by RT activity, but not Gag expression and alters the intracellular distribution of *gag-pol* vRNA support a model in which NPM1 plays an active role in the cytoplasmic localization and utilization of *gag-pol* vRNA and possibly, gRNA packaging. However, whether the functional involvement of NPM1 in *gag-pol* vRNA localization and utilization occurs in the cytoplasm, nucleus, or nucleolus still requires investigation. Furthermore, examination of the molecular mechanism by which NPM1 associates with the *gag-pol* vRNA/RNP is necessary.

One intriguing mechanism for NPM1 and gRNA packaging has been proposed by the Parent group. They have identified a NoLS in the NC domain of Rous Sarcoma Virus (RSV) Gag and HIV-1 Gag, and show that a fraction of expressed Gag localizes to the nucleolus (207). Furthermore, they demonstrate that NPM1 co-localizes with HIV-1 Gag in the nucleolus and HIV-1 Rev and HIV-1 Gag localize to the nucleolus when co-expressed, thus suggesting the nucleolus could play a contributing role to HIV-1 gRNA packaging and virus assembly (207). However, these results are in disagreement with others that report HIV-1 Gag does not undergo nuclear trafficking (14, 137). Thus, further studies are needed to address these discrepancies.

Another possible post-transcriptional mechanism for NPM1 could be an effect on gRNA translation, more specifically, ribosomal frameshifting and the maintenance of the *gag: gag-pol* ratio. The exact mechanism for HIV-1 ribosomal frameshifting has yet to be resolved; however, the proposed model supports the hypothesis that frameshift

efficiency can be altered when specific aspects of the translation elongation process are modified. Thus, cellular factors such as ribosomal proteins, translation elongation and termination factors, kinase and phosphotase factors that modulate the activity of translation, and RNA-binding proteins that can remodel RNA structures, may contribute to ribosomal frameshift modulation (24, 181, 311). NPM1 has several cellular activities that are regulated through the phosphorylation, acetylation, ubiquitylation and SUMOylation of its functional domains. Specifically, NPM1 has been demonstrated to bind both DNA and RNA, promote RNA processing, act as a molecular chaperone, interact with several RNA processing factors, as well as interact with both the large and small ribosomal subunits (205, 210, 241, 277). NPM1 has not been investigated as a cellular factor in ribosomal frameshifting to date and, currently, only one cellular factor has been reported to affect HIV-1 frameshifting fidelity, Eukaryotic release factor 1 (eRF1) (181). Nonetheless, increasing or decreasing HIV-1 gRNA frameshift efficiency has been demonstrated to cause a decrease in RT activity and virus infectivity (90, 160, 181, 287). Furthermore, *Dulude et. al.* demonstrated that a decrease in frameshift efficiency, caused by introducing mutations within the slippery sequence and the frameshift stimulatory signal, correlated with equivalently reduced viral infectivity and replication kinetics but not Gag processing (90). Analysis of Gag processing in infectious mutant virions revealed processing defects of various extents, thus lacking proportionality between frameshift efficiency (90). My findings that depletion of cellular NPM1 inhibits HIV-1 production but not Gag expression could support a role for NPM1 in frameshifting efficiency. Although I did not detect a decrease in viral infectivity in the

experiments reported in this thesis, it is important to note that viral infectivity was determined by infecting cells with equivalent RT units and not p24 quantity, thus essentially normalizing for infectivity. A more accurate examination of viral infectivity would be to infect cells with equivalent p24, which should be performed. Interestingly, in the p24 ELISA experiments of macrophage supernatants, I did not detect a statistically significant difference in p24 activity, suggesting that VLPs could be released. Thus, further experimentation is required to determine whether infectivity of the virus is affected. Moreover, to examine whether a frameshift increase or decrease is observed in NPM1 depleted cells, examination of Pol, RT, PR and/or IN protein detection needs to be examined as well as Northern blot analysis of vRNA species should be completed.

To further demonstrate a role for NPM1 in HIV-1 replication, I examined whether virus production could be restored in cells depleted of NPM1 expression through stable cell lines expressing siRNA-resistant forms of GFP-NPM1 proteins. However, despite being resistant to siNPM1 treatment, the stable cell lines were unable to achieve wildtype levels of virus production. In Chapter 4, I present some possible explanations for why this phenotype was observed, with improper fusion protein folding being the most probable. However, it is important to address whether I could be observing an off-target effect from the siRNA duplexes used the experiments presented in this thesis. The likely candidate to be targeted by my siNPM1 duplexes is CLEC2D. Strikingly, CLEC2D mRNA has a 94% mRNA sequence identity to NPM1 when aligned (www.ncbi.nlm.nih.gov). Even more outstanding is all TRC lentiviral NPM1 shRNAs available on Open Biosystems can also target CLEC2D (1). In fact two of the shRNAs

available are listed for both NPM1 and CLEC2D (TRCN0000062268 and TRCN0000062270). CLEC2D is a cell membrane spanning protein that functions as a CD161 ligand and has been shown to be expressed on some hematopoietic cells (including macrophages and T cells), osteoblasts, and chondrocytes (122, 126). It is important to note, CLEC2D expression was not found on cells isolated from patients with high HIV-1 viral loads in the only study to date examining this relatively uncharacterized protein (126). Nonetheless, I examined whether CLEC2D mRNA was detectable in HeLa cells and whether siNPM1 treatment affected CLEC2D expression in the absence and presence of virus. Reverse transcriptase quantitative real-time polymerase chain reaction (RT-qPCR) analysis was used to compare expression levels of the targeted cDNA. For a detailed description of the experimental method, I refer the reader to Chapter 2, Materials and Methods. I was unable to detect any expression of CLEC2D cDNA for all conditions in my RT-qPCR experiments, thus supporting the premise CLEC2D has no role in the results reported in this thesis. Moreover, initial RT-qPCR results examining NPM1 cDNA expression in siNPM1 treated cells show specific depletion of NPM1 (Figure A1-8). However, to corroborate a role for NPM1 function in HIV-1 replication, performing parallel experiments with alternative siRNAs directed against NPM1 gene expression would strengthen the significance of my results.

In addition, to further explore the role of NPM1 as a HIV-1 *gag-pol* vRNA post-transcriptional modulator and address whether NPM1 has global and/or off-target effects on cellular mRNA expression, examination of NPM1 depletion on HIV-1 vRNA mRNA expression profiles is needed and is currently in progress. HIV-1 vRNA and cellular

GAPDH mRNA expression will be examined using a modified RT-qPCR method described by Stolarchuk *et al*., followed by analysis of the ratio of spliced (2 kb), partially spliced (4 kb) and unspliced (9 kb) classes of vRNA species expressed (295). This will be the first study of its kind and the results could elucidate possible mechanisms for NPM1 function in HIV-1 replication.

5.5 Concluding Remarks

In this thesis, I present findings that identify distinct functional roles for the nucleolus and NPM1 in HIV-1 replication (Figure 5-2). More specifically, my research findings support a model for a Rev-independent nucleolar localization step of intron-containing vRNA prior to export. My results also show NPM1 does not participate in Rev nucleolar localization or Rev-mediated vRNA export, as previously proposed. Instead, I provide evidence to support a new role for NPM1, the cytoplasmic localization and utilization of gRNA. The findings herein thus provide exciting and novel insight to assist in resolving the complexities of HIV-1 vRNA export and expression and enhance our current understanding of HIV-1 biology.

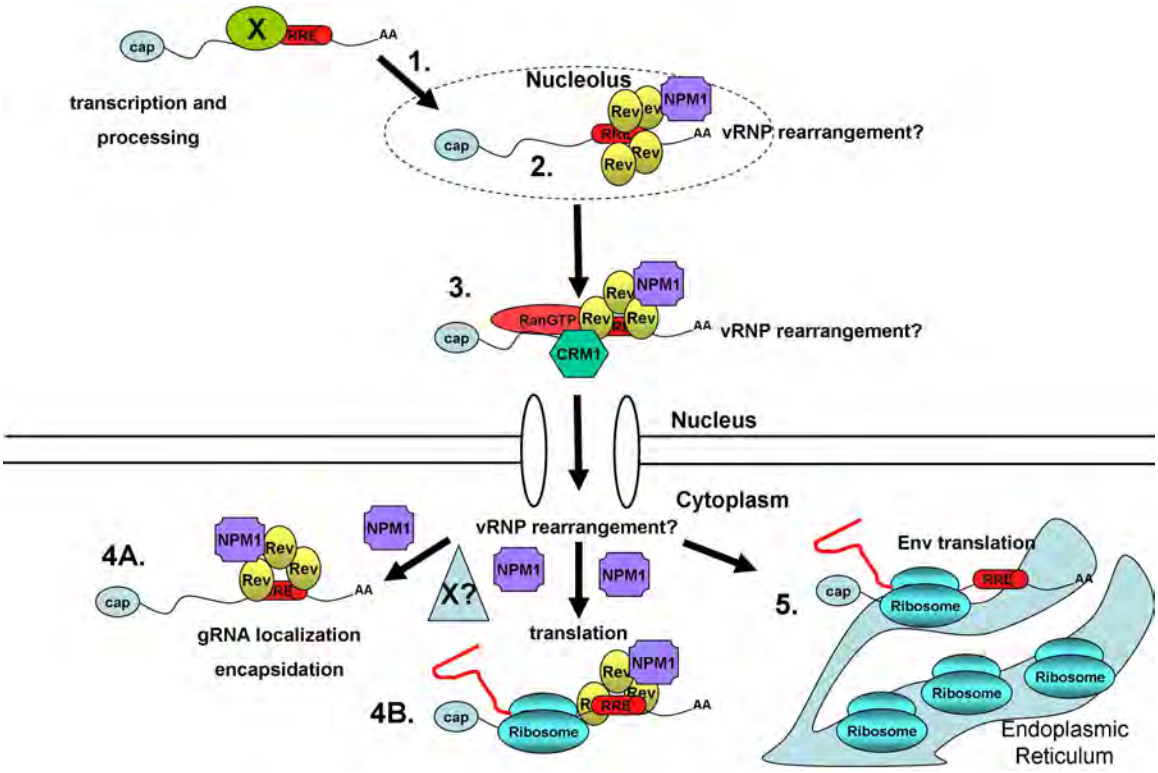


Figure 5-2. HIV-1, the Nucleolus and NPM1: A Potential Model. (1.) After transcription and processing intron-containing vRNAs are transported to the granular component of the nucleolus by cellular factor(s) (designated as X) and independent of HIV-1 Rev. (2.) Trafficking to the nucleolus allows for Rev-mediated export complex formation. (3.) HIV-1 Rev mediates intron-containing vRNA export from the nucleus. (4A.) Full-length *gag* vRNA is localized for encapsidation (gRNA) or (4B.) translated on on cytosolic ribosomes to produce Gag and Pol polyproteins. (5.) *env* vRNA is translated on ER associated ribosomes. A possible vRNP rearrangement to include NPM1 occurs to influence downstream full-length, gRNA localization and/or utilization. However, whether NPM1 has this effect in nucleolus (2.), nucleus (3.) or in the

cytoplasm (4A. and 4B.) needs to be determined. Furthermore, whether NPM1 associates directly with the gRNA, with gRNA and HIV-1 Rev, or with another factor (shown as X?) in the absence or presence of Rev to orchestrate gRNA localization and/or utilization requires investigation.

APPENDIX

I conceived and performed all experiments presented in this appendix.

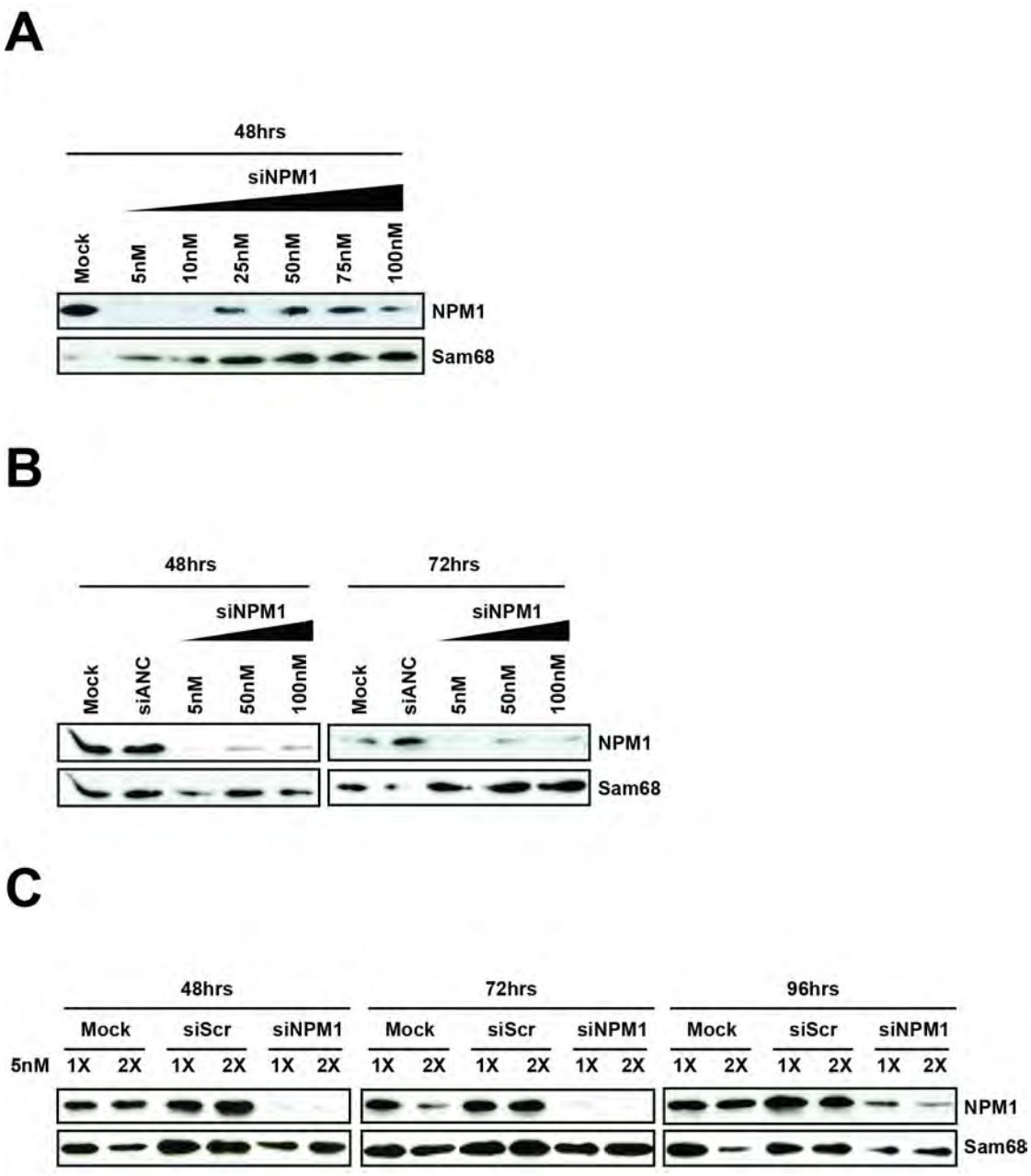


Figure A1-1. Efficient depletion of NPM1 expression by RNAi is achieved in HeLa cells at a concentration of 5 nM, with a maximum reduction occurring at 72hrs post siRNA treatment. (A) HeLa cells were transfected with reagent alone (Mock) or

increasing concentrations of siNPM1. At 48hrs post siRNA transfection, cell lysates were analyzed for NPM1 gene-specific knock-down by Western blotting with an anti-NPM1 antibody. Sam68 was detected with an anti-Sam68 antibody as a total protein loading control. (B) HeLa cells were transfected with reagent alone (Mock), 100 nM of siANC or increasing concentrations of siNPM1. At 48- and 72hrs post siRNA transfection, cell lysates were analyzed for NPM1 gene-specific knock-down by Western blotting with an anti-NPM1 antibody. Sam68 was detected with an anti-Sam68 antibody as a total protein loading control. (C) HeLa cells were transfected with reagent alone (Mock), 5 nM of a control NPM1 scrambled siRNA (siScr) or 5 nM siNPM1 once (1X) or twice (2X) as described in Materials and Methods. At 48-, 72-, and 96hrs post siRNA transfection, cell lysates were analyzed for NPM1 gene-specific knock-down by Western blotting with an anti-NPM1 antibody. Sam68 was detected with an anti-Sam68 antibody as a total protein loading control.

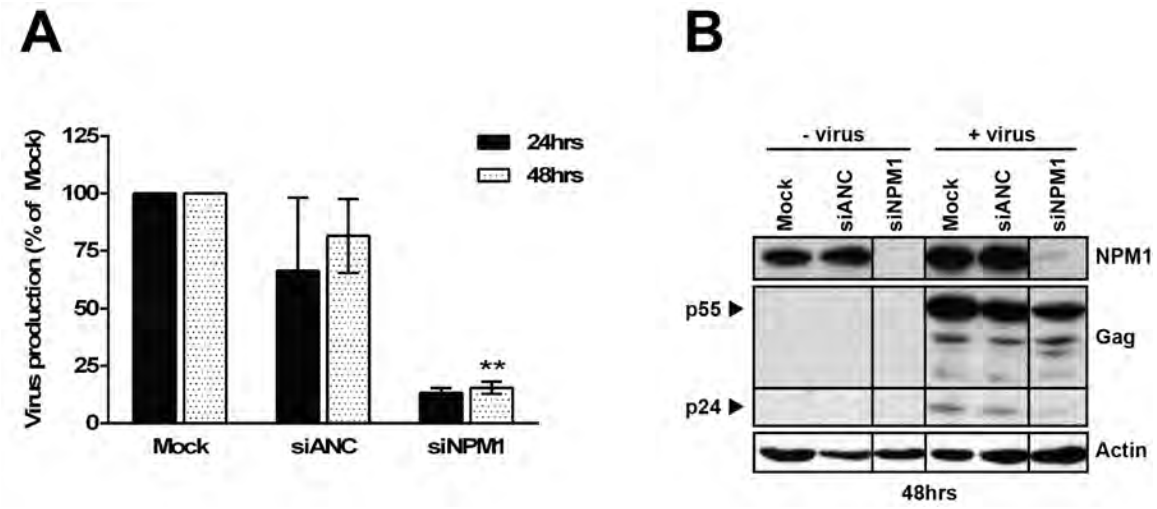


Figure A1-2. Depletion of NPM1 expression by RNAi inhibits HIV-1 production in transiently transfected HeLa cells. HeLa cells were transfected with reagent alone (Mock), 100 nM of siANC or 100 nM of siNPM1. At 48hrs post siRNA transfection, cells were transfected with reagent alone or with a pNL4-3 proviral clone. (A) Virus production was determined by quantifying viral RT enzyme activity of culture supernatants 24- and 48hrs post infection as described in Material and Methods. Bars represent the total mean percent of virus production from four independent experiments ± SEM. Multiple student T-test analysis determined a non significant P value at 24hrs (P= 0.145) and a significant value at 48hrs (**, $P \leq 0.01$). (B) Cells lysates were analyzed for NPM1 gene-specific knock-down and intracellular Gag expression by Western blotting 48hrs post transfection with an anti-NPM1 and anti-Gag antibody, respectively. Data shown is representative of cell lysates prepared from experiments in A.

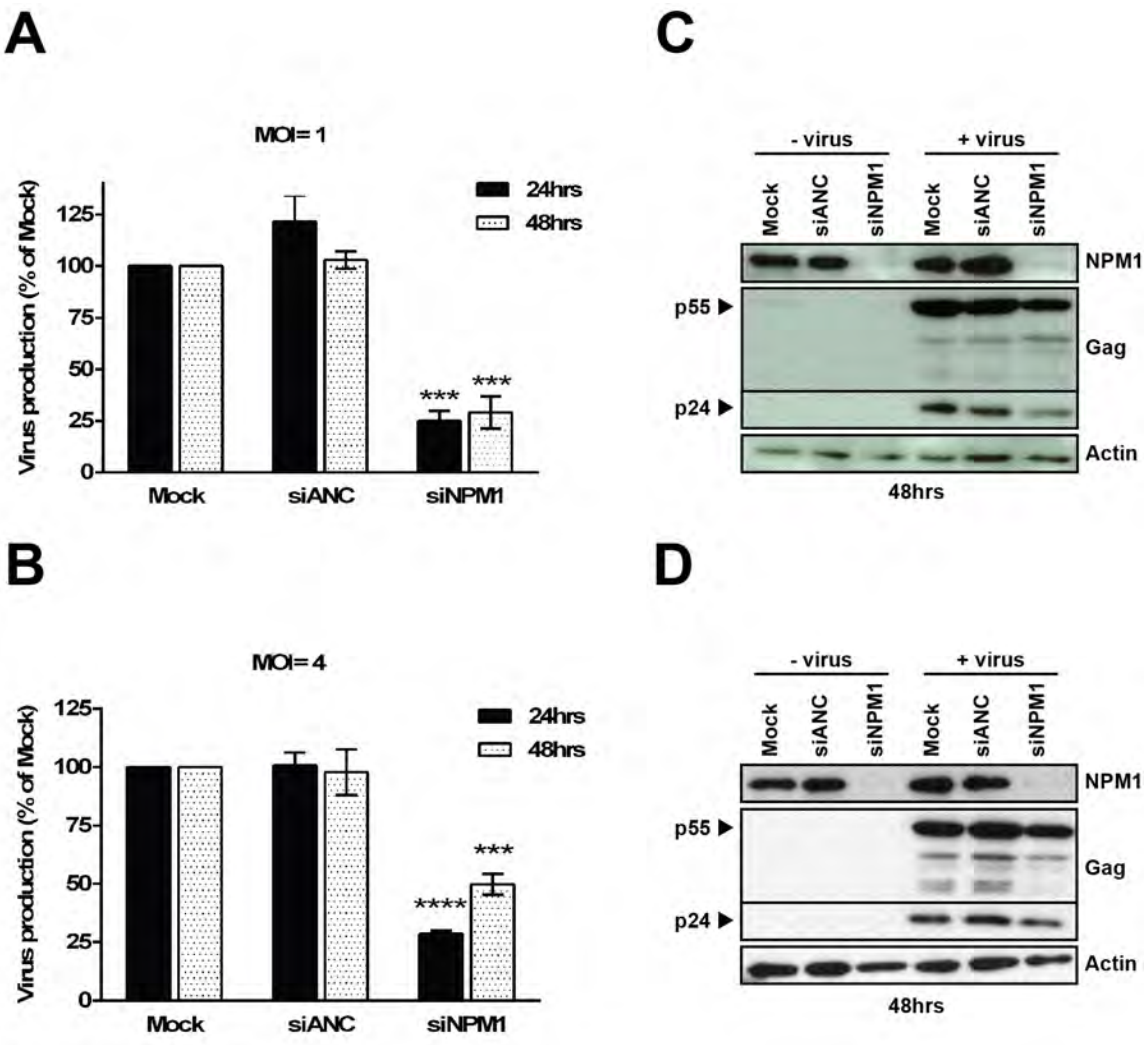


Figure A1-3. Depletion of NPM1 expression by RNAi inhibits HIV-1 production similarly in HeLa cells infected at different MOIs. HeLa cells were transfected with reagent alone (Mock), 5 nM of siANC or 5 nM of siNPM1. At 48hrs post siRNA transfection, cells were uninfected or infected with VSV-G pseudotyped NL4-3 virus at an MOI of 1 or 4. (A, B) Virus production was determined by quantifying viral RT enzyme activity of culture supernatants 24- and 48hrs post infection as described in

Material and Methods. Bars represent the total mean percent of virus production from four independent experiments, two performed in duplicate ± SEM (A) or five independent experiments, four performed in duplicate ± SEM (B). Multiple student T-test analysis determined significant P values at both an MOI of 1 (A; ***, P≤ 0.001) and an MOI of 4 (B; ***, P ≤ 0.001; ****, P≤ 0.0001). (C, D) Cells lysates were analyzed for NPM1 gene-specific knock-down and intracellular Gag expression by Western blotting 48hrs post infection with an anti-NPM1 and anti-HIV-1 Gag antibody, respectively. Data shown is representative of cellular lysates prepared from experiments in A (C) and B (D).

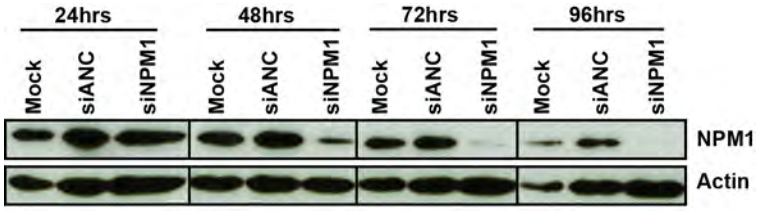


Figure A1-4. Efficient depletion of NPM1 expression by RNAi is achieved at a concentration of 10 nM in monocyte-derived primary human macrophages, with maximum reduction occurring at 96hrs post siRNA transfection. MDM were transfected with reagent alone (Mock), 10 nM of siANC or 10 nM of siNPM1 as described in Materials and Methods. Cell lysates were analyzed for NPM1 gene-specific knock-down by Western blotting 24-, 48-, 72- and 96hrs post transfection with an anti-NPM1 antibody. Actin was detected with an anti-Actin antibody as a total protein loading control. Data shown represents one experiment.

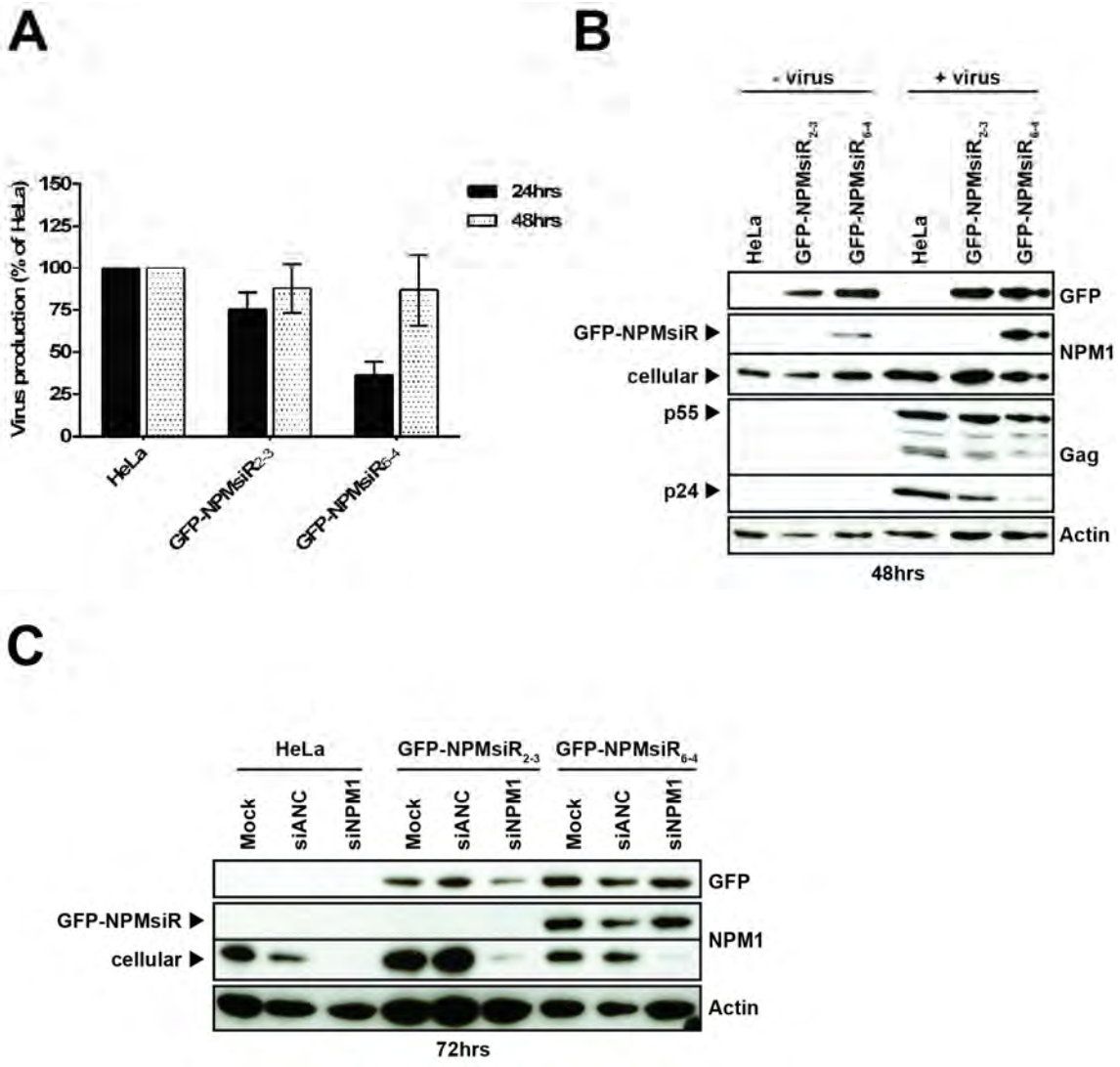


Figure A1-5. GFP-NPMsiR stable cell lines support variable levels of HIV-1 production and intracellular GFP-NPMsiR protein expression. (A, B) HeLa and HeLa-derived stable cell lines were uninfected or infected with VSV-G pseudotyped NL4-3 virus at an MOI of 4. (A) Virus production was determined by quantifying viral RT enzyme activity of culture supernatants 24- and 48hrs post infection as described in Material and Methods. Bars represent the total mean percent of virus production from

three independent experiments ± SEM. (B) Cell lysates were analyzed for GFP-NPMsiR, NPM1 and intracellular Gag expression by Western blotting 48hrs post infection expression with an anti-GFP, anti-NPM1 and anti-Gag antibody, respectively. Data shown is representative of cell lysates prepared from experiments in A. (C) HeLa or GFP-NPM1siR stable cell lines were transfected with reagent alone (Mock), 5 nM of siANC or 5 nM of siNPM1. At 72hrs post siRNA transfection, cells were harvested, lysed and were analyzed for GFP-NPMsiR and NPM1 expression by Western blotting with an anti-GFP and anti-NPM1 antibody.

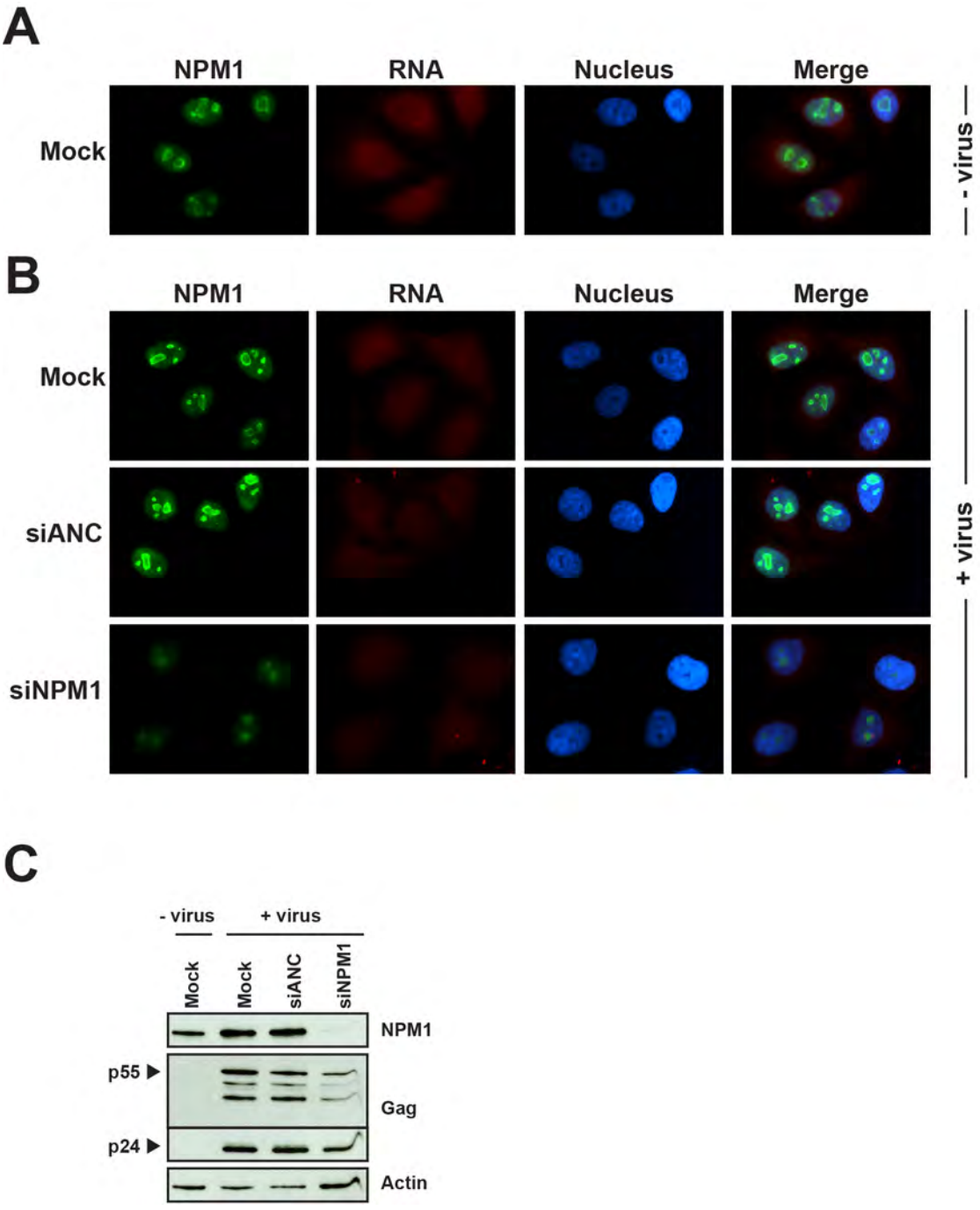


Figure A1-6. Depletion of NPM1 expression by RNAi has no discernable effect on GAPDH RNA localization. (A, B) HeLa cells were transfected with reagent alone

(Mock; A, B top panel), siANC (B middle panel) or siNPM1 (B lower panel). At 48hrs post siRNA transfection, cells were uninfected (A) or infected with VSV-G pseudotyped NL4-3 virus at an MOI of 4 (B). At 24hrs post infection, cells were fixed with 4% paraformaldehyde/5 mM $MgCl_2$/1X PBS and analyzed using coupled FISH- IF. A Cy3-conjugated GAPDH RNA-specific probe was used to detect cellular GAPDH mRNA (red). Immune detection of NPM1 (green) was performed using anti-NPM1 primary antibody and an AF-488 conjugated secondary antibody. The nucleus (blue) was detected by DAPI. Data shown is representative of multiple fields examined from two independent experiments. (C) HeLa cell lysates prepared from experiments in A and B were analyzed for NPM1 gene-specific knock-down and intracellular Gag expression by Western blotting with an anti-NPM1 antibody and anti-Gag antibody, respectively.

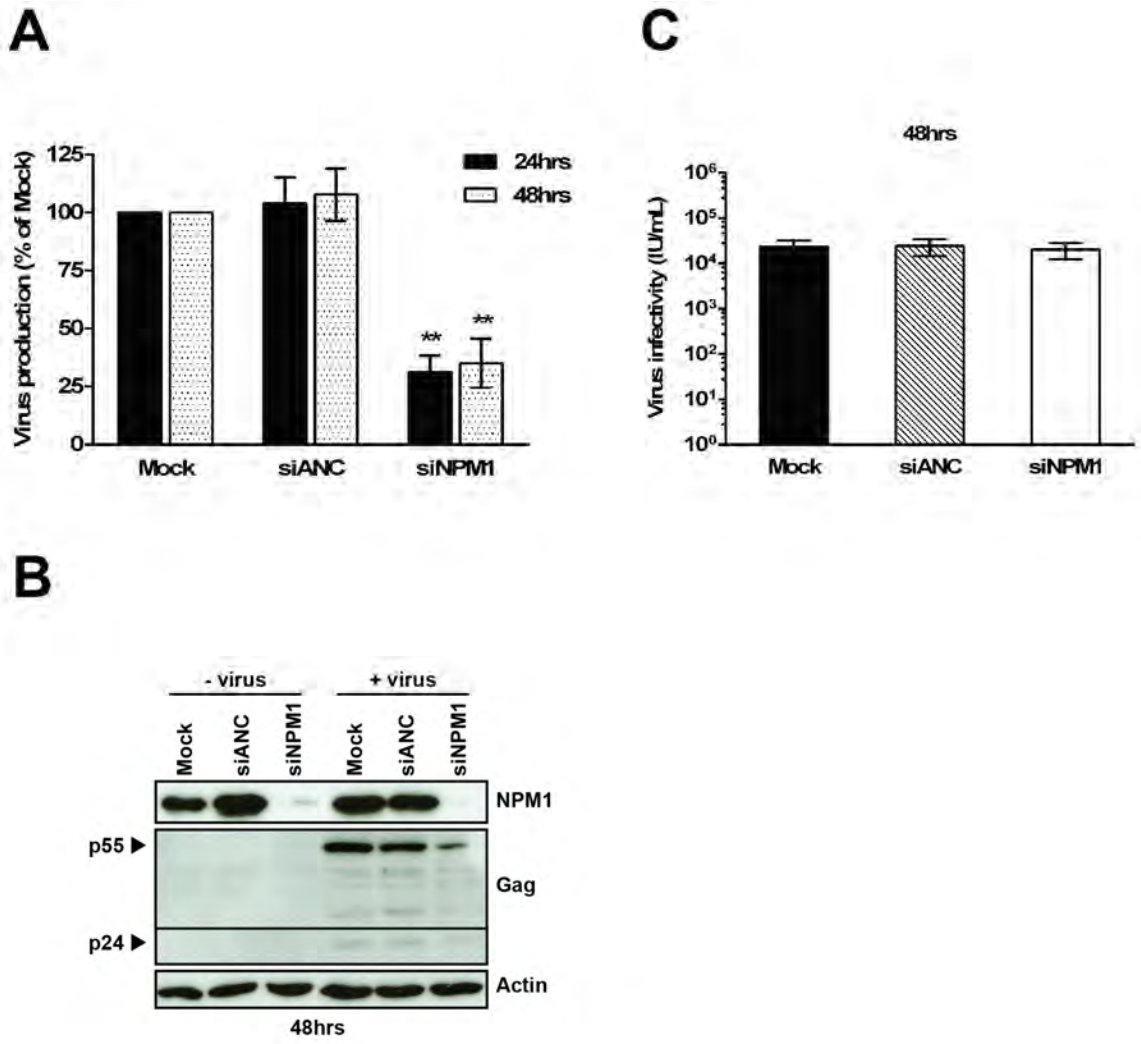


Figure A1-7. Depletion of NPM1 expression by RNAi has no discernable effect on HIV-1 infectivity. HeLa cells were transfected with reagent alone (Mock), 5 nM of siANC or 5 nM of siNPM1. At 48hrs post siRNA transfection, cells were uninfected or infected with VSV-G pseudotyped NL4-3 virus at an MOI of 1. (A) Virus production was determined by quantifying viral RT enzyme activity of culture supernatants 24- and 48hrs post infection as described in Material and Methods. Bars represent the total mean

percent of virus production from two independent experiments performed in duplicate ± SEM. Multiple student T-test analysis determined significant P values at 24- and 48hrs (**, P≤ 0.01). (B) Cells lysates were analyzed for NPM1 gene-specific knock-down and intracellular Gag expression by Western blotting 48hrs post infection with an anti-NPM1 and anti-HIV-1 Gag antibody, respectively. Data shown is representative cell lysates prepared from experiments in A. (C) The infectivity of virus present in culture supernatants collected 48hrs post infection was determined using TZM-bl indicator cells and monitoring β-gal expression. Aliquots of supernatants containing equivalent RT activity units were serially diluted 10-fold in antibiotic-free media. Duplicate cell monolayers were inoculated with the diluted virus cultures. At 48hrs post infection, cells were washed, fixed and assayed for β-gal enzymatic activity as described in Materials and Methods. Bars represent the total mean virus infectivity of culture supernatants collected from experiments in A ± SEM. A random-block one way ANOVA analysis determined non significant P values for virus infectivity (P= 0.1556).

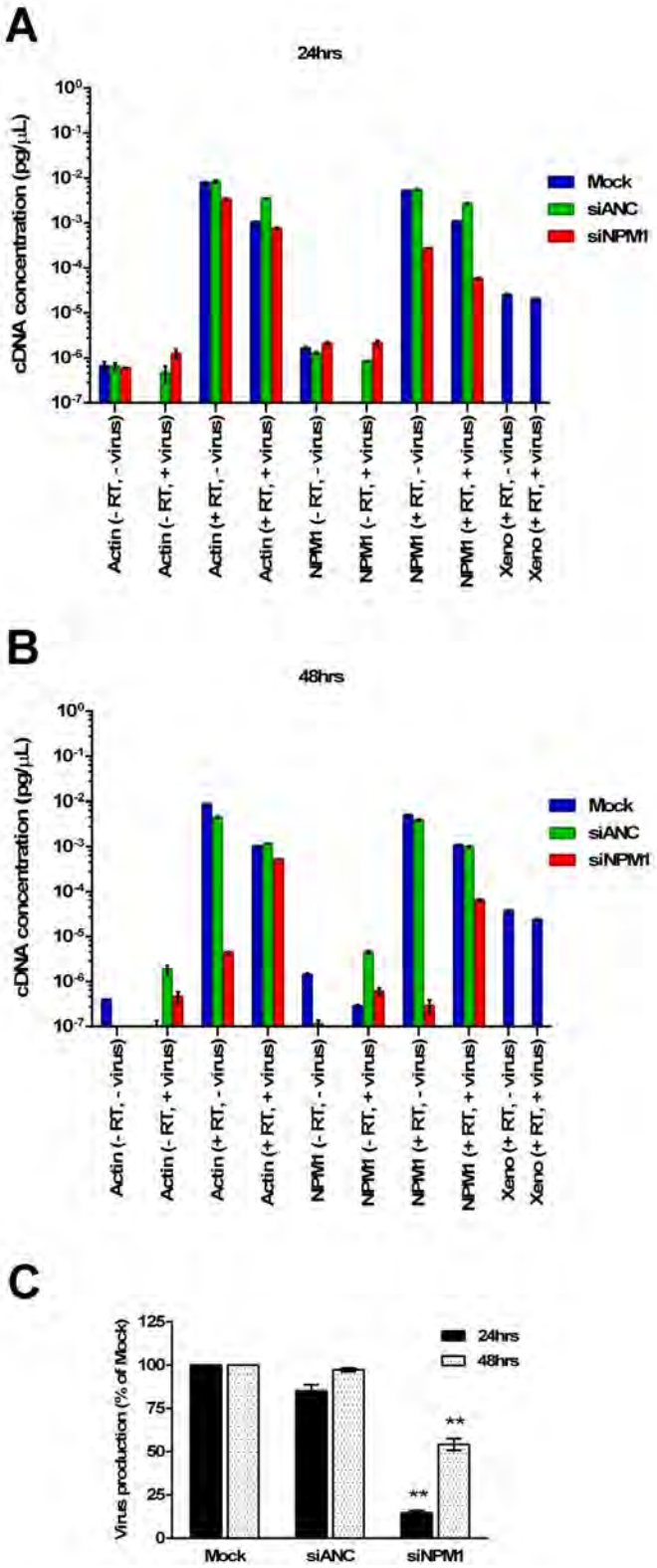

A
24hrs
cDNA concentration (pg/μL)
Mock
siANC
siNPM1
Actin (- RT, - virus)
Actin (- RT, + virus)
Actin (+ RT, - virus)
Actin (+ RT, + virus)
NPM1 (- RT, - virus)
NPM1 (- RT, + virus)
NPM1 (+ RT, - virus)
NPM1 (+ RT, + virus)
Xeno (+ RT, - virus)
Xeno (+ RT, + virus)
B
48hrs
cDNA concentration (pg/μL)
Mock
siANC
siNPM1
Actin (- RT, - virus)
Actin (- RT, + virus)
Actin (+ RT, - virus)
Actin (+ RT, + virus)
NPM1 (- RT, - virus)
NPM1 (- RT, + virus)
NPM1 (+ RT, - virus)
NPM1 (+ RT, + virus)
Xeno (+ RT, - virus)
Xeno (+ RT, + virus)
C
Virus production (% of Mock)
24hrs
48hrs
**
**
Mock
siANC
siNPM1

Figure A1-8. RT-qPCR analysis confirms efficient RNAi-mediated depletion of NPM1 mRNA in the absence or presence of HIV-1 infection. (A, B) HeLa cells were transfected with reagent alone (Mock), siANC or siNPM1. At 48hrs post siRNA transfection, cells were uninfected or infected with VSV-G pseudotyped NL4-3 virus at an MOI of 4. Cells were harvested 24hrs (A) and 48hrs (B) and total RNA was prepared for RT-qPCR analysis as described in Materials and Methods. Life Technologies Gene Expression Assay primers were used to quantify control gene β-actin (Actin), NPM1 and the XenoRNA (Xeno) control target expression levels in the absence (-) or presence (+) of virus. To confirm mRNA/ cDNA-specific amplification, samples were analyzed in the absence of reverse transcriptase (- RT). (A, B) Bars represent the mean of one experiment, assayed in duplicate ± SEM. (C) HeLa cell supernatants from A and B were analyzed for virus production by quantifying viral RT enzyme activity as described in Material and Methods. Bars represent the total mean percent of virus production from two independent experiments ± SEM. Multiple student T-test analysis determined significant P values at 24- and 48hrs (**, $P \leq 0.01$).

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